U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Analysis of Venom in Cape Verde Cone Snails

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Tese de Doutoramento apresentada à
Faculdade de Ciências da Universidade do Porto,
Ciência e Tecnologia do Ambiente

2016

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2016

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Acknowledgments

To Professor Vitor Vasconcelos for the extraordinary support, constant availability and the amazing encouragement. A special thanks for giving me the opportunity to learn and contribute to science.

To my co-supervisor Professor Agostinho Antunes for the availability, valuable suggestions and motivation.

To Professor Baldomero M. Olivera, for the extraordinary guidance and for giving me the opportunity to work and learn in his laboratory at University of Utah – USA; I would like to thank specially Julita S. Imperial for her daily guidance and encouragement. Thanks to all Olivera Lab. Staff, specially: My Huynh, Terry Merrit, Joseph W. Aman and Samuel S. Espino. Thanks to Zhenjian Lin, Eric W. Schmidt, David Morgenstern and Beatrix Ueberheide.

To all LEGE – Pedro Leão, Micaela Vale, João Morais, José Carlos, Vitor Ramos, Marisa Silva, Marisa Freitas, Ana Regueiras – and CIIMAR team members for the encouragement and incredible friendship over the years and also for the technical and administration support.

A special thanks to all my family, particularly to my parents, Maria Hirondina Brito Neves and João Baptista Neves because they always believed in me.

I am especially grateful to my wife, Ana Sofia, for the amazing and unconditional support. I dedicate this thesis to her and our son, David Neves.

This work was supported by *Fundação para a Ciência e a Tecnologia* (FCT) - PhD Scholarship (SFRH/BD/51477/2011); and the follow project: MARBIOTECH (NORTE-07-0124-FEDER-000047); NOVELMAR **-** Novel marine products with biotechnological applications (NORTE-01-0145-FEDER-000035); and FCT project UID/Multi/04423/2013.

Preamble

This thesis is based on the law (*artigo 8º* from *Decreto Lei nº 388/70; República Portuguesa*), where most of the chapters are results of published works including work in collaboration. The candidate participated on the acquisition, analysis, discussion and preparation of the work for publication.

The field work was done by the candidate through snorkeling and Scuba diving (until 20 meters deep), with the support of CIIMAR, *Fundação Calouste Gulbenkian and* University of Cape Verde (UniCV). The work presented here was conducted, in most part, in CIIMAR (Center of Marine and Environmental Research, University of Porto), but the candidate also conducted work for this dissertation during a 9-month stay at Department of Biology (University of Utah, Salt lake City, USA). The candidate has also established work partnerships with Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Institute of Chemistry and Biology Technology (ITQB) at New University of Lisbon, and New York University Langone Medical Center, Department of Biochemistry and Molecular Pharmacology (New York, USA).

Thesis Organization

This dissertation was organized on five chapters. Chaper 1 presents a general introduction to the Cone Snail distribution, diversity, biology, ecology, structure and activity of toxins (conotoxins).

Chaper 2 describes the identification of 12 peptides from *C. crotchii* using MALDI-TOF and mass-matching. The number of molecular masses studied resembles the outputs from other studies performed in the general *Conus* enabling to validate the approach. It was identified several disulfide-rich conotoxins in *C. crotchii* venom duct samples that belongs to O1-superfamily (Eb6.18, Leo-O2, Bu2, PVIIA), A-superfamily (im23.3, Ai1.2, PnMGMR-02), T-superfamily (Ca5.1, TxVA) and O2- (Ec15a), O3- (VnMSGL-0123) and D-superfamilies (VxXXB).

In Chaper 3 it is described the collection and first toxinological characterization of the *C. ateralbus* specie. An excitatory activity that acted on a majority of mouse lumbar dorsal root ganglion neurons was purified and characterized from *C. ateralbus* venom. The 30AA venom peptide was named δ-conotoxin AtVIA. AtVIA has conserved sequence elements when compared to δ-conotoxins from fish-hunting *Conus* species, and from a peptide purified from *Conus tessulatus*, a species in the worm-hunting Indo-Pacific clade Tessiliconus.

In Chaper 4 it is identified a novel small-molecule guanine derivative with unprecedented features. Genuanine was isolated from the venom of two cone snail species (*C. genuanus* and *C. imperialis*). Genuanine causes paralysis in mice, indicating that small molecules and not just polypeptides may contribute to the activity of cone snail venom.

Finally, in Chapter 5 it is presented the overall discussion of results and main conclusions.

The structure of the manuscripts and the scientific paper was maintained according to the journal guidelines in which they were published or submitted, including the reference style.

Abstract

The cone snails (genus *Conus*), living in the tropical habitats, are highly venomous predatory gastropods that use peptide toxins (conotoxins) for major environmental interactions, such as prey capture, defense and competition. Approximately 800 species of *Conus* are known and each one can express more than 100 different peptides. Conotoxins (disulfide-rich peptides), can potentially target components of the neuromuscular system, mainly ligand- and voltage-gated ion channels.

The study of *Conus crotchii* venom duct revealed about 488 molecular masses between 700 and 3000 Da. Those masses were searched by matching with known peptide sequences from UniProtKB (UniProt Knowledgebase) protein sequence database. Through this method we were able to identify 12 conopeptides. For validation it was considered the error between the experimental molecular mass (monoisotopic) and the calculated mass of less than 0.5 Da. All conopeptides detected belong to the A-, O1-, O2-, O3-, T- and D-superfamilies, which can block Ca^{2+} channels, inhibit K^{+} channels and act on nicotinic acetylcholine receptors (nAChRs). Only a few of the detected peptides have a 100% UniProtKB database similarity, suggesting that several of them could be newly discovered marine drugs.

Conus ateralbus is an endemic cone snail that has been found only on the west side of the island of Sal, in the Cape Verde Archipelago off West Africa. It was described the collection and first toxinological characterization of this species. An excitatory activity was purified and characterized from *C. ateralbus* venom that acted on a majority of mouse lumbar dorsal root ganglion neurons. This 30AA venom peptide, δ-conotoxin AtVIA, has conserved sequence elements when compared to δ-conotoxins from fish-hunting *Conus* species, and from a peptide purified from *Conus tessulatus*, a species in the worm-hunting Indo-Pacific clade *Tessiliconus*. In contrast, there is no comparable sequence similarity with δ-conotoxins from the venoms of molluscivorous *Conus* species. A rationale for the presence of δ-conotoxins that are potent in vertebrate systems in two different lineages of worm-hunting cone snails is discussed.

On the study of *C. genuanus* venom it should be noted that small molecules also contribute to the neuroactivity of the venoms. A novel guanine derivative, genuanine, causes paralysis in mice and is found in the venoms of at least two cone snail species.

The global results show that the venoms of Cape Verde cone species are rich source of powerful bioactive molecules in vertebrate systems and clearly present stronger pharmacology interest.

Keywords: *Conus*, conopeptides, conotoxins, genuanine.

Resumo

Os caracóis marinhos (do gênero *Conus*) que se encontram em habitats tropicais são gastrópodes predadores altamente venenosos que utilizam toxinas peptídicas (conotoxinas) para as principais interações ambientais, como a captura de presas, defesa e competição. Cerca de 800 espécies de *Conus* são conhecidas e cada uma pode expressar mais de 100 péptidos diferentes no veneno. As conotoxinas (péptidos ricos em ligações dissulfureto) podem atingir potencialmente os componentes do sistema neuromuscular, principalmente os canais iónicos.

No estudo do ducto do veneno de *Conus crotchii* foram pesquisados cerca de 488 massas moleculares entre 700 Da e 3000 Da. Fez-se uma pesquisa dos peptídeos com os já conhecidos a partir do banco de dados UniProtKB (UniProt Knowledgebase). Através deste método, identificaram-se 12 conopéptidos. Para a validação foi considerado o erro (menos de 0,5 Da) entre a massa molecular experimental (monoisotópico) e a massa calculada. Todos os conopéptidos detectados pertencem às superfamílias A-, O1-, O2-, O3-, T e D-, que podem bloquear os canais de Ca^{2+}, inibir canais de K^{+} e actuar sobre os receptores nicotínicos da acetilcolina (nAChRs). Apenas alguns dos péptidos detectados partilham 100% de similaridade com os dados da UniProtKB, sugerindo que vários compostos detectados podem ser novas toxinas marinhas.

Conus ateralbus é um cone caracol endémico que foi encontrado apenas no lado oeste da ilha do Sal, no arquipélago de Cabo Verde (África Ocidental). Foi descrita a recolha e a primeira caracterização toxicológica desta espécie. Uma atividade excitatória foi caracterizada a partir do veneno purificado do *C. ateralbus* que atuou sobre a maioria dos neurónios dos ratos - *dorsal root ganglian* (DRG). O peptídeo caracterizado possui 30AA, designado de δ-conotoxin AtVIA, e conservou elementos da sequência quando comparado a δ-conotoxins de espécies *Conus* que se alimentam de peixes, e quando comparado a um peptídeo purificado a partir de *Conus tessulatus*, uma espécie do Indo-Pacífico que se alimenta de vermes (*clade* Tessiliconus). Em contraste, não há nenhuma similaridade de sequência comparável com δ-conotoxin AtVIA dos venenos de espécies *Conus molluscivorous*. É discutida a razão para a presença de δ-conotoxin AtVIA que são potentes em sistemas de vertebrados em duas linhagens diferentes dos *Conus* do grupo *worm-hunting*.

No estudo do veneno de *C. genuanus* apresenta-se que as moléculas pequenas também contribuem para a actividade neuronal dos venenos. Um derivado novo da guanina, genuanine, causa paralisia em ratos e é encontrado em venenos de pelo menos duas espécies de caracóis cone (*C. genuanus* e *C. imperialis*).

Os resultados na generalidade mostram que as espécies de *Conus* de Cabo Verde são importantes fontes de moléculas bioactivas que podem afectar os sistemas dos vertebrados, o que leva a um forte interesse farmacológico.

Palavras-chave: *Conus*, conopeptides, conotoxins, genuanine.

Contents

List of Tables

Chapter 5: Discussion and Conclusions

List of Figures

Chapter 1: Introduction

Chapter 2: Conopetides from Cape Verde *Conus crotchii*

Chapter 3: Characterization of a δ-Conotoxin from *Conus ateralbus*, a Vermivorous Cone Snail from the Cape Verde Archipelago

Chapter 4: Small Molecules in the Cone Snail Arsenal

List of Abbreviations

2DE gel	Two-dimensional gel electrophoresis
AA	Amino Acids
Ach	Acetylcholine
ACN	Acetonitrile
BLAST	Basic Local Alignment Search Tool
C.	*Conus*
CIIMAR	Center of Marine and Environmental Research of the University of Porto
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance Spectroscopy
COSY	Correlation Spectroscopy
DIPEA	*N*,*N*-diisopropylethyl amine
DMF	Dimethylformamide
DRG	Dorsal Root Ganglion
DTT	Dithiothreitol
ECD	Eletron Capture Dissociation
ESI	Electrospray Ionization
ETD	Electron Transfer Dissociation
FDA	United States Food and Drug Administration
^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRESIMS	High-resolution electrospray ionisation mass spectrometry
HSQC	Heteronuclear Single Quantum Coherence
IAA	Iodoacetamide

IACUC	Institutional Animal Care and Use Committee
IC	Intracranial injection
IP	Indo-Pacific region
IPATIMUP	Institute of Molecular Pathology and Immunology of the University of Porto
ITQB	Institute of Chemistry and Biology Technology
LC	Liquid Chromatography
LEGE	Ecotoxicology, Genomics and Evolution Laboratory
MALDI	Matrix-assisted Laser Desorption Ionization
MASCOT	Matrix Science protein identification program
MS	Mass Spectrometry
MTBE	Methyl-*tert*-butyl ether
MTSET	[2-(Trimethylammonium)ethyl] methane thiosulfonate bromide
MTS-R	Methanethiosulfonate reagents
nAChRs	Nicotinic Acetylcholine Receptors
NCBI	National Center for Biotechnology Information
NOESY	Nuclear Overhauser effect spectroscopy
NMP	*N*-methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance Spectroscopy
NSS	Normal Saline Solution
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RP	Reversed-Phase
SDS	Sodium Dodecyl Sulfate
TFA	Trifluoroacetic Acid
TOF	Time Of Flight
PAGE	Polyacrylamide Gel Electrophoresis

PTM	Post-translational Modifications
UniCV	University of Cape Verde
UniProtKB	UniProt Knowledgebase
UV	Ultra-violet

Chapter 1

Introduction

1. Introduction

1.1. Cone Snails (*Conus*): A brief history

Cone snails are tropical marine gastropods, belonging to the Conidae family (superfamily Conoidea) and the genus *Conus* (Figure 1). Although in recent taxonomic assessments marine gastropods have been separated into multiple families that belong to the Conoidea superfamily, they are close to other venomous marine gastropods like Terebridae and Turridae [1,2].

The Conoideans comprise a major and diverse group of gastropods with over 3000 species of venomous marine snails. Fossil evidence of cone snails (*Conus*) was known in Lower Eocene approximately 55 million years ago (first radiation), and in England and France (land flora indicates tropical climatic conditions). A second radiation occurred in Miocene, followed by a decrease in the Pliocene and then a very rapid expansion has continued until present [2].

Figure 1: Shells of some of the cone snails belonging to the CIIMAR collection. From left to right, top row: *C. marmoreus*; *C. maldivus*; *C. viridulus*. Second row: *C. textile*; *C. miles*; *C. praelatus*. Third row: *C. tessulatus*; *C. omaria*; *C. quercinus*. Bottom row: *C. terebra*. The shells shown are not represented to scale.

Recent literature has suggested the existence of approximately 700 species of *Conus* [1–6,8–10]. In fact, *Conus* are one of the largest single genera of living marine invertebrates [11], and an important contributor to biodiversity in the sea. Cone snails are commonly found and extensively distributed (Figure 2) throughout all tropical oceans. They live frequently on stones with some sand and algae, in bays or harbors with shallow and clean waters. In the Indo-Pacific region (IP) approximately 60% of the *Conus* species occur, being the location in the world where they predominate [12]. Only in Papua New Guinea – known to be a biodiversity "hot spot" for marine benthic invertebrates [13] –, about 80 species are known, and there are roughly 70 species in China [14]. In Brazil (Western Atlantic) there are around 18 species of *Conus* [15] , and in Southeast Africa 84 species are reported but only 18 are endemic [16]. All *Conus* are venomous and use this venom for self-defense and prey capture [1,17,18]. The biological activity of *Conus* venom shows great diversity, and molecular studies of venom components open a window for biomedicine investigation.

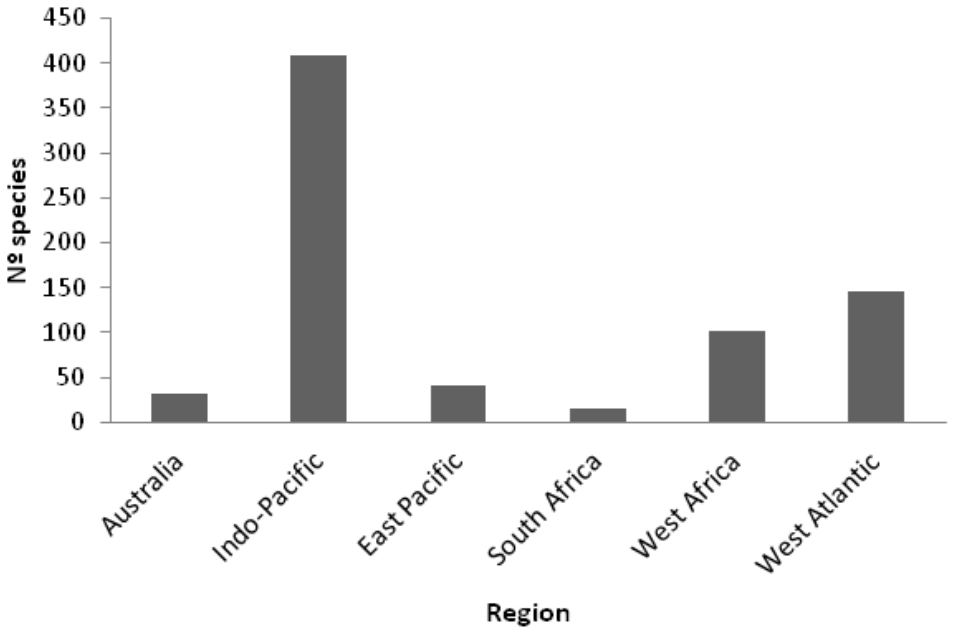

Figure 2: Cone species distribution per region (data adapted from the Illustrated Catalog of the Living Cone Shells 2013) [19].

1.2. Cone Snails (*Conus*): Biology and Ecology

Cone snails are found in all tropical marine ecosystems, being prominent around coral reefs and shallow tropical marine habitats. In Indo-Pacific coral reefs it is possible to find over 30 different species of *Conus* [13]. The diversity of species outside the tropics

is poor, although some species have adapted to colder waters. The type of habitat is widely varied, including for example in sand or beneath and between the rocks and algae. *Conus* eggs (Figure 3) can be found under or over the rocks, and some species are collected only at depths around 150 meters [2,13]. Despite the fact that most cone snails are nocturnal and have two eyestalks, their vision is poor. However, *Conus* species have a great chemosensory prowess and this is used to effectively track prey [20].

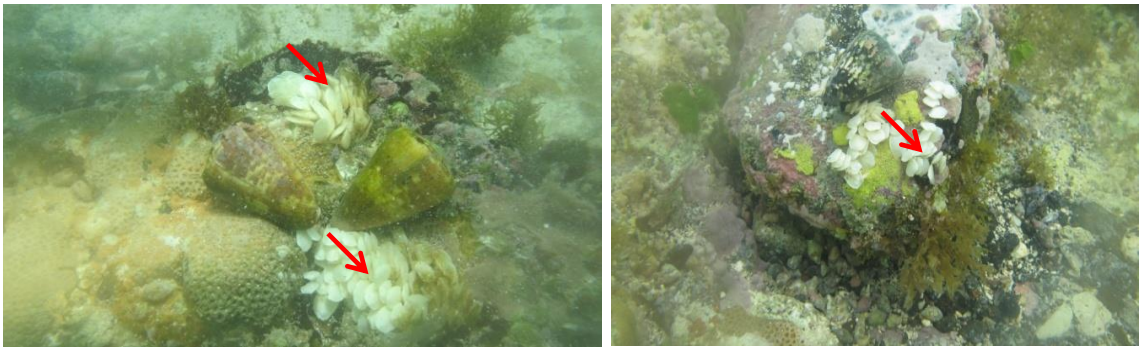

Figure 3: **From left to right, *Conus venulatus* on egg capsules (red arrow), 5-6 m depth, Boa Vista Island; *Conus ateralbus* on egg capsules, 2-3 m depth, Sal Island; Cape Verde arquipelago.**

Depending on its diet and other factors, each species of cone snails has a preferred environment. They are very slow moving species that will typically stay in the same location for most of its lifetime. One of the classifications of *Conus* is related to their major prey. Cone snails are classified into three groups: the (1) vermivorous species that eat polychaetes, hemichordates, and echiuroid worms (worm-hunting), being this the largest group 75%; (2) molluscivorous species that hunt other mollusks (snail-hunting); and (3) piscivorous cone snails (fish-hunting) [2,5,21–27] that hunt fish comprising around 10% of the species. The piscivorous *Conus* have the most potent toxins and are considered the most dangerous, with lethal effects on humans [18].

Few species with mixed diets, such as *Conus californicus* have been reported [28]. For prey capture the cone snails use their powerful venom, although the venom is also used for other environmental interactions such as a defense weapon, and competitor deterrence [20].

Some of the fish-hunting species (e.g. *Conus geographus*) are clearly dangerous to humans. In fact, the geography cone (*Conus geographus*) has been responsible for around three-dozen human fatalities [2].

1.2.1. Cone Snails Venom Apparatus

All cone snails use venom to capture prey and for other interactions such as defense from predators. As previously reported, cone snails are classified according to their type of prey (vermivorous, molluscivorous, and piscivorous), which is related to the biological diversity of the venom, usually a complex cocktail of potent and specifics peptides. Generally, each cone snail hunts only one kind of prey using its venom. The venom has more than 100 different peptides in each *Conus* species [29], showing species-specific differences in terms of prey, predators and competitors.

The biological system of the *Conus* venom apparatus (e.g. the venom apparatus of a fish-hunting *C. striatus,* Figure 4) consists of the venom bulb, the venom duct (where the venom is secreted), and lastly the radular sheath (radular teeth) followed with harpoon-like radula [2]. All cone snails have general anatomical similarities of the venom apparatus.

As opposed to the gastropod radula (usually with rows of chitinous teeth that scrape or bite during feeding), in cone snails the radular teeth are free and resemble barbed hypodermic needles very important for the venom injection into the prey, and also as a harpoon to tether fish [30]. When they look for prey, a single harpoon tooth is transferred into the lumen of a long distensible proboscis. When the extended proboscis feel that the prey is very close, the tooth is transferred from the radular sac to the proboscis. The venom is pressed by the muscle bulb (venom bulb) [31].

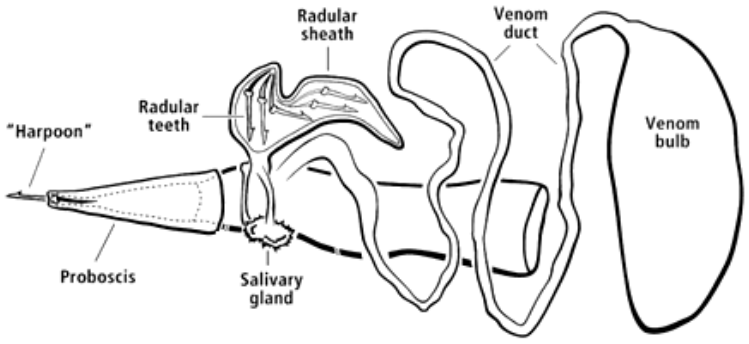

Figure 4: ***Conus*** **venom apparatus of the** ***C. striatus*****. Reproduced by personal permission from Baldomero M. Olivera [2]. This venom apparatus also represents the general cone snail venom apparatus.**

In terms of the radular teeth, *C. californicus* for example, an atypical member of the genus *Conus*, is considered a generalized feeder with unusual radular teeth. The

anatomic radular teeth of *Conus* are so different for each hunting group that have been used for *Conus* taxonomy [2,30,32,33].

However, the venom duct, considered to be a very complex structure, has sparked discussion and some diverging interpretation. The venom duct produces the biologically active component (conotoxins) in epithelial cells lining the tubular venom duct. From production to venom it is considered the synthesis, processing and packaging of conotoxins; the production is followed by storage of radular teeth and the transfer to the tip of the proboscis; in the end the insertion of the tooth with ejection of venom [30,34]. The venom duct is divided into proximal and distal duct according to the influence in venom production. These general production steps are relatively invariable between *Conus* species, however there are clear differences in how some *Conus* species attack their prey. In the next sections we will analyze the closer relationship among proximal and distal duct, the venom production, and the influence into cone snails environmental behavior. We will also focus on conotoxins composition, envenomation strategies, developments and how this influences their molecular and pharmacological classification.

1.2.2. *Conus*: Envenomation Strategies

Here we will analyze the closer relationship among proximal and distal duct, the venom production and its influence into cone snails behavior. The successful prey capture depends on the venom potential, rapid physiological and biomechanical mechanisms [33]. However, there are some different ways for prey capture that are worth being discussed as some *Conus* attack quicker than others.

Contrary to the vermivorous and piscivorous *Conus* species that usually use a single radular tooth, the molluscivorous inject more than one tooth into a single prey [2]. *Conus ammiralis* (mollusc-hunting) has been observed to harpoon its prey more than half a dozen times [1]. Also, molluscivorous cone snails, such as *C. victorae* injected five teeth and take about one hour to hunt a single prey snail (*Cantharus erythrostomus*). Their behavior in complex feeding process was first described in detail for vermivorous and piscivorous, between 1956 and 1971 [35]. The vermivorous *C. betulinus* is described to eat large polychaetes, and cone snail took more than half an hour to engulf the entire polychaete (may have in some cases over a meter long) [1]. Although the vermivorous *Conus* are considered the largest group, the venoms of fish-

hunting *Conus* when tested in vertebrates are much more lethal than those of mollusk- and vermivorous-hunting *Conus* [2].

The conotoxins potential of the fish-hunting *Conus* has been extensively studied and some different envenomation strategies are clearly noted. The *Conus catus* (fish-hunting), for example, take around 1 milliseconds to use their radular tooth to capture its prey in “one shot” [33]. The ecological behavior at feeding time led the fish-hunting cone snails into two classes: the “hook-and-line” fishing snails (e.g. *Conus striatus*) and “net-fishing” cone snails (e.g. *Conus geographus*) [36]. Olivera review (2002) shows these two distinct strategies for *Conus striatus* and *Conus geographus* when they hunt fish. The strategy of *Conus striatus* (mostly at night when fish are less active) is to bury itself in the sand, with only their fins extended to suddenly attack a fish by extending their long nose (proboscis) that acts like a harpoon line and strikes the fish. The prey moves freely within a small area, than is injected with venom through the hollow radular tooth (like as a hypodermic needle) [2]. After the fish jerks violently it is immobilized. The second strategy, used by *Conus geographus*, is to surround and completely cover the fish with its highly distensible false mouth like a large net. The fish are then stung within the rostrum one by one. Aquarium observations showed that *Conus geographus* never buries itself, it prefers to hide in crevices of rocks or coral rubble [2,37]. The group of toxins responsible for this behavior is called toxin “cabal” [37]. Listed at least two types of cabals: (a) the “lightning-strike cabal”: use e. g. by *C. striatus*, these conotoxins cause almost-immediate titanic paralysis causing immobilization in a short time; (b) the “nirvana cabal”: associated with the behavior of the *C. geographus* usually causes flaccid paralysis in fish [2]. The strategy for prey capture is species-specific and is related to aspects of venom biochemical and pharmacological diversity [20]. Ten years after approval of the pharmacological conotoxins ω-MVIIA (United States Food and Drug Administration, FDA, 2004), commercial name Prialt®, an interesting study was able to identify the distinctive use of *Conus* venom in response to predation or defensive situation and the relationship with the proximal (posterior) and distal portion of the venom duct (see Dutertre *et al*., 2014) [18]. Using two fish-hunting cone snails, *C. geographus* and *C.mamoreus*, Dutertre *et al.* (2014) reported that the venom duct has two distinguishable regions of toxin production, designated as defence-evoked (proximal sections, nearest the venom bulb) and predation-evoked (distal sections, nearest the pharynx) [18]. The two distinguished regions produce two different types of toxins. Notably, *C. geographus* contains high levels of paralytical toxins in the defence-evoked section, while the distinguished predation-evoked venom, contains toxins

inactive at to human target, used usually for prey capturing. These results can explain the lethal effect of *C. geographus* in humans, by using the most potent toxins for defence and this results in the blockage of neuromuscular receptors [18,38,39]. However, the mechanism for "moving" the 'defence-evoked' venom is unclear, because the defense toxins use the same venom duct of the predation venom. The question is, how does the venom "travel" through the venom duct from proximal region to the proboscis? This important question requires further investigation.

The evolution of envenomation strategies has been demonstrated in snakes [40], as a classically predatory adaptation. However, the defense is very important for animal survival. Regionalization of toxins production was reported in other venomous animals such as sea anemone and scorpions [41,42]. Venom regionalization may explain the offense and defense action. Some venomous animal use the most toxic part of venom if their life is in danger [18,41–44]. Molecular evolution of predatory and defensive toxins showed evidence of positive selection, namely in A- and O-superfamily conotoxins [45,46].

1.3. *Conus* Venom Peptides

Conus venoms – also termed conotoxins or conopeptides – have been referenced in the literature as a large group of peptides. Every *Conus* species contains about 100-200 distinct toxins (small venom peptides) [2,47]. By definition, conotoxins are small peptides usually with 12–35 amino acids, in general connected through one to four disulfide bridges. But as is found in larger proteins, these compounds exhibit many motifs [16,22,29,48–56]. Conotoxins are usually defined by disulfide-rich peptides (two or more disulfide), but on the classification of *Conus* venom there are also disulfide-poor peptides (one or none disulfide bond) [28]. However, most of the scientific work developed has been on disulfide-rich conopeptides (conotoxins). A Pubmed search of conotoxins demonstrated the existence of about 2833 peer reviewed articles, but only 118 referring to conopeptides (http://www.ncbi.nlm.nih.gov/pubmed/?term=Conotoxins; on 2014 – 11 – 16).

On the chemical structures of the conotoxins, the cysteine frameworks are quite predictable although they have great sequence diversity. This is clear for example on ω-conotoxins MVIIA (isolated from the *Conus magus*, that targets the N-type Ca^{2+} channels) and GVIA (isolated from the *Conus geographus*, that targets the N-type Ca^{2+} channels) where in the mature toxin region, 16 of the 22 non cysteine residues are

different resulting in a 73% divergence [36]. The cysteine framework in general is given by the formula (X)n1$\mathbf{C_iC_{ii}}$(X)n2$\mathbf{C_{iii}}$(X)n3$\mathbf{C_{iv}}$(X)n4 for the majority of the conotoxins, where the number of amino acids in n2 and n3 can have profound consequences in their selectivity toward a specific receptor class, and also this affects the conotoxin classification in terms of conotoxins family (bioactivity) [57]. For example, in the family α-conotoxins it is unambiguous that with a 3/5 cysteine framework (n2=3 and n3 = 5) the conotoxins will target muscle nAChRs, while conotoxins with a 4/7 framework (n2 = 4 and n3 = 7) will be specifically neuronal nAChRs [58–60].

Conotoxins contain numerous post-translational modifications (PTM). PTM of amino acids (AA), are covalent modifications characteristically pertaining to side chains or "R" group functions of the common AA. PTM (Table 1) are proteolytic processing of propeptide to mature peptide, including the following: C- and N-terminal modification, disulfide bridge formation, hydroxylation, carboxylation, bromination, epimerization, cyclization, sulfation and O-glycosylation [28,29,57,61–64].

Table 1: Post-translational modifications (PTM) of some *Conus* peptides

PTM	Peptide	*Conus* species
C- Terminal modification	BtX	*C. betulinus*
N- Terminal modification	TxVIA	*C. textile*
Hydroxylation	μ-GIIIA	*C. geographus*
Carboxylation	Conatokin-G	*C. geographus*
Bromination	vc5c	*C. victoriae*
Epimerization	r11a	*C. radiatus*
Sulfation	PnIA	*C. pennaceus*
O-glycosylation	tx5a	*C. textile*

Post-translational modifications on conotoxins molecular structure are responsible for a rich chemical diversity and may provide great pharmacology diversity (receptor selectivity) of the cone snail venom [57,62].

Overall, conotoxins have the following classification: (a) gene superfamily, classification center on evolution relationships between conopeptides; (b) cysteine framework, classification according to the arrangement of cysteines; and (c) pharmacological family, classification system that reflects the physiologically relevant protein target [28]. The predatory cone snails are very small animals that use their potent venoms as a major weapon. Conotoxins are considered underlying for discovery of ligands with

subtype selectivity for many classes and families of receptors and ion channels [18,57,65].

1.3.1. Conotoxins: Superfamilies and families

The classification of Cone snail venom (conotoxins or conopeptide) includes the gene superfamilies and also pharmacological families. Conopeptide precursors consisting of a highly conserved signal region (~20 amino-acids) followed by a protein precursor (more variable) and the formation of hypervariable mature toxin that have only a few conserved amino acids (disulfite-bonded cysteine) [66]. Peptides from *Conus* species share a highly conserved signal sequence in their precursors and a conserved arrangement of cysteine residues (Cysteine framework) they belong to the same superfamily [67]. In the last 10 years several superfamilies have been characterized [48]. So far, 26 conotoxin superfamilies were identified: A-,B-, C-, D-, I1-, I2-, I3-, J-, L-, M-, O1-, O2-, O3-, P-, S-, T-, V-, X1-, X2-, X3-, X4-, X5-, X6-, X7, G and Y-superfamilies. Some superfamilies have small amino acid variation and with that new branches, as the superfamily B (B1, B2 and B3), the superfamily I (I1, I2 and I3), the superfamily O (O1, O2 and O3) and X-superfamily (X1 to X7) [28,66,68]. The largest gene-superfamily in the recent data from Conoserver (www.conoserver.com, on December 2014) and belongs to the "top 4" (Table 2) are: O- superfamilies, being identified 575 protein precursors O1, 133 protein precursors O2 and 43 protein precursors O3; M- superfamily, with 443 protein precursors; A- superfamily, with 286 protein precursors and T- superfamily, with 239 protein precursors. The proteins identified in these four superfamilies represent 87% of the peptides present in the species that have been identified. On the other hand, the conotoxin families have an impressive diversity of targets ranging from voltage-gated ion channels (sodium, calcium, and potassium) to ligand-gated ion channels (such as nicotine receptors and serotonin receptors) [69].

Table 2: Conotoxin classification by gene superfamilies and pharmacological families. For each gene superfamily the cysteine pattern and their target, the pharmacology families, and the reference are represented

Superfamily	Cysteine framework	Pharmacological families	Mode of action (target)	*Conus* specie (e.g. peptide)	Sequence	Reference
M-superfamily	CC-C-C-CC	*κ*	K^+ channels	*C. radiatus* (RIIIK)	LOS**CC**SLNLRL**C**OVOA**C**KRNO**CC**T*	[25]
	CC-C-C-CC	μ	Na^+ channels	*C. geographus* (GIIIA)	RD**CC**TOOKK**C**KDRQ**C**KOQR**CC**A*	[70]
	CC-C-C-CC	*ψ*	nAChR	*C. purpurascens* (PIIIE)	HOO**CC**LYGK**C**RRYOG**C**SSAS**CC**QR*	[71]
A-superfamily	CC-C-C	α	nAChR	*C. aulicus* (AuIB)	G**CC**SYPP**C**FATNPD**C***	[20]
	CC-C-C-C-C	αA	nAChR	*C. ermineus* (EIVA)	G**CC**GPYONAA**C**HO**C**G**C**KVGROOY**C**DROSGG*	[72]
	CC-C-C-C-C	*KA*	K^+ channels	*C. striatus* (SIVA)	ZKSLVPSVITT**CC**GYDOGTM**C**OO**C**R**C**TNS	[28]
T-superfamily	CC-CC	χ	NE transporter	*C. marmoreus* (MrIA)	NGV**CC**GYKL**C**HO**C**	[73]
O-superfamily	C-C-CC-C-C	ω	Ca^{2+} Channels	*C. magus* (MVIIA)	**C**KGKGAK**C**SRLMYD**CC**TGS**C**RSGK**C***	[74]
	C-C-CC-C-C	K	K^+ channels	*C. purpurascens* (PVIIA)	**C**RIONQK**C**FQHLDD**CC**SRK**C**NRFNK**C**V	[75]
		δ	Na^+ channels	*C. catus* (CVIE)	YG**C**SNAGAF**C**GIHPGL**CC**SEL**C**LVW**C**T	[76]
		μO	Na^+ and Ca^{2+} channels	*C. marmoreus* (MrVIA)	A**C**RKKWEY**C**IVPIIGFIY**CC**PGLI**C**GPFV**C**V	[31]

* COOH-terminal group is amidated.

The superfamilies are subdivided into conotoxin families also previously termed ‘‘pharmacological families’’ according to their molecular targets [66]. The O-superfamily is considered a large and diverse group of peptides, sharing a cysteine pattern (C–C–CC–C–C, with three disulfide bridges) and appears as the most diverse in terms of pharmacological function. As pharmacological peptides family include μO- conotoxins that block voltage-gated sodium channels, ω-conotoxins that block voltage-sensitive calcium channels, κ-conotoxins that block voltage- gated potassium channels, and δ-conotoxins that delay the inactivation of voltage-sensitive sodium channels families as members [20,52,77,78]. The O-superfamily peptides can be found in all major feeding types of *Conus* but most typically in fish-hunting and mollusk-hunting *Conus* species [22,78]. Several peptides of O-superfamily are extensively used as molecular probes in neuroscience and pharmacology research where it belongs to the only peptide previously approved for the treatment of chronic pain by the FDA [9].

The M-superfamily of conotoxins is considered the most diverse of all the conotoxin superfamilies yet characterized [25], share Cys pattern as ‘‘–CC–C–C–CC–’’. Due to its great diversity, this superfamily can be divided into five branches as M-1, M-2, M-3, M-4 and M-5, according to the different residue number in the last Cys loop, mean according to the number of amino acids that exist between the fourth and fifth cysteine. In terms of pharmacology family the biological activities include block voltage-gated sodium (μ) and potassium (κM) channels and acetylcholine receptors (ψ) [25]. Considered important because they allow for the development of therapeutic treatments, this superfamily is found in all of the hunting *Conus* species [79].

The A-superfamily peptides consists of four distinct pharmacological families, which are the κA-, ρ-, αA- and α–conotoxins. Alfa (α)-conotoxins have four cysteines (CC-C-C) and are nicotinic acetylcholine receptors (nAChRs) antagonists, αA-conotoxins also target nAChRs, but they have the CC-C-C-C-C cysteine framework. Rho (ρ)-conotoxins are preferential a_{1B}-adrenoceptor antagonists while κA-conotoxins (CC-C-C-C-C) are thought to target K^+ channels [48,77,79,80].

Finally, T-superfamily conotoxins have been identified in the venom ducts of different cone snails, including fish-hunting, snail-hunting, and worm-hunting *Conus* [81], and are extremely small peptides (10–17 amino acids in length). The T-superfamily, includes μ-, χ-, and ϵ-conotoxins families are members [76,81–83]. The widespread distribution of T-superfamily conotoxins suggests that they might have important physiological functions for the genus. For example, the highly post-translationally modified (ε-conotoxin TxIX) of Tx5a from *C. textile* elicits hyperactivity and spasticity in

mice when injected intracranially [84]. The T-superfamily conotoxins includes two types of peptides, which share two common cysteine pattern; (a) T-1-conotoxins group with cysteine pattern CC–C–C (also named τ-CTX) and (b) cysteine pattern CC–C–C (χ-CTX) [85,86].

Since the ω-conotoxin MVIIA, purified from *Conus magus* was approved by FDA in December 2004, the investigation of these beautiful and intriguing animals increased significantly. The *Conus* peptides that share a highly conserved signal sequence have been organized into gene superfamilies, for example, O- M- A- T- superfamilies. Also, peptide toxins form a large family (according to the biologic activity) from cone snail venoms that act on a broad spectrum of ion channels and receptors. Due to their enormous diversity and target specificity, cone snails peptides have become inestimable tools in molecular pharmacology and as therapeutic agents. Despite the clear pharmacological potential, the production and the biology envenomation mechanism remains unclear, this reinforces the need to increase the level of research in this subject. Most of the venom studies belong to the piscivorous species. However, vermivorous and molluscivorous species show an interesting role in pharmacology and have more species belonging to these two groups.

1.4. Aims of the study

Of the 57 *Conus* species described in Cape Verde archipelago, 54 are endemic, of which only three are non-endemics [87]. In Cape Verde archipelago the great variety of *Conus* species represents about 10% of species diversity throughout the world. Most of the studies conducted so far concern the morphology, phylogeny and philogeography [88]. In this context it was projected the analysis and identification of conotoxins. To achieve this goal the specific aims proposed were: (1) identification of toxins (e.g. conopeptides); (2) analyze the diversity of toxins; and (3) search for potential bioactive substances produced by *Conus* from Cape Verde.

With this work, the specific proposed aims were achieved mainly with the comprehensive study of three species, *C. crotchii* (Chapter 2), *C. ateralbus* (Chapter 3) and *C. genuanus* (Chapter 4).

1.5. References

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Chapter 2

Conopetides from Cape Verde *Conus crotchii*

2. Conopetides from Cape Verde *Conus crotchii*

Mar. Drugs **2013**, *11*, 2203-2215; doi:10.3390/md11062203

OPEN ACCESS
Marine Drugs
ISSN 1660-3397
www.mdpi.com/journal/marinedrugs

Article

Conopeptides from Cape Verde *Conus crotchii*

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Abstract

Marine Cone snails of the genus *Conus* contain complex peptide toxins in their venom. Living in tropical habitats, they usually use the powerful venom for self-defense and prey capture. Here, we study *Conus crotchii* venom duct using a peptide mass-matching approach. The *C. crotchii* was collected on the Cape Verde archipelago in the Boa Vista Island. The venom was analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). About 488 molecular masses between 700 Da and 3000 Da were searched by matching with known peptide sequences from UniProtKB protein sequence database. Through this method we were able to identify 12 conopeptides. For validation we considered the error between the experimental molecular mass (monoisotopic) and the calculated mass of less than 0.5 Da. All conopeptides detected belong to the A-, O1-, O2-, O3-, T- and D-superfamilies, which can block Ca^{2+} channels, inhibit K^{+} channels and act on nicotinic acetylcholine receptors (nAChRs). Only a few of the detected peptides have a 100%

UniProtKB database similarity, suggesting that several of them could be newly discovered marine drugs.

1. Introduction

Cone snails (genus *Conus*) are venomous predators belonging to the Conidae family. There are ~700 *Conus* species, all carrying complex arrays of peptide toxins in their venom [1]. *Conus* species normally live in tropical habitats of shallow water, on sand or near coral reefs and may cause lethal paralysis to their prey. Considered aggressive predators, cone snails are usually classified, depending on their prey, into three groups: vermivorous (worm-hunting), molluscivorous (other gastropods-hunting), and piscivorous cone snails (fish-hunting). However, some *Conus* can feed on hemichordates, bivalve mollusks and echiuroids, but a few species are considered generalist (e.g., *C. californicus*). *Conus* can be also dangerous to humans. The fish-hunting species *C. geographus*, have caused about three dozen fatalities in human poisoning cases [2,3]. The venom bioactive molecules, known as conopeptides or conotoxins (indiscriminate using of this work), are used to capture prey, as self-defense from predators or to prevent competition [4,5]. Some authors have estimated that there are 100–200 distinct peptides per species, but recent work suggested that more than 1000 distinct conopeptides may be found per species [6]. These venoms mainly include linear peptides (usually disulfide-rich) and powerfully folded mini-proteins [7], exhibit various neuropharmacological properties with special incidence on ion channels and receptors [8,9]. Therefore, conopeptides have been considered powerful tools in neuroscience, and, for example, in December 2004, the synthetic version of the peptide ω-conotoxin MVIIA (commercial name Prialt®; Elan Pharmaceuticals, Inc., Dublin, Ireland) from *C. magus* has been approved by the United States Food and Drug Administration (FDA) to treat chronic pain in humans [10–13]. Despite this achievement, the overall knowledge of *Conus* venom proteins and peptides is scarce compared to other animal venom-producers (e.g., snakes, scorpions, spiders and sea anemones), thus providing a huge potential for the discovery of new pharmacological drugs [14]. The majority of *Conus* venom mini-proteins have a sequence length of 12–35 amino acids, normally with a high incidence of post-translational modifications. There are four classifications: (i) disulfide-rich conopeptides (conotoxins), which have two or more disulfides bridges, and disulfide-poor conopeptides, with one or none disulfide bond (conopressins, contryphans, conantokins, and contulakins); (ii) “gene superfamily” scheme that share a highly conserved sequence; (iii) “cysteine framework” scheme sorts them according

to the arrangement of cysteines; and (iv) “pharmacological family” scheme reflects the target specificity of each conopeptide [2,9,10,15].

For the analysis of peptides the matrix-assisted laser desorption/ionization equipped with a time-of-flight (MALDI TOF/TOF) mass analyzer has been one of the most valuable analytical tools. This technique is relatively easy to perform (user friendly), is reliable and at the same time enables high-throughput sample analyses. Through MALDI-TOF, valuable data is generated [16], which contributes to the rapid discovery and characterization of new *Conus* marine drugs. The masses of peptides produced can be compared and “matched” to know sequences available on databases (e.g., UniProtKB and ConoServer). This methodology has been used to make available the identity of any protein whose full-length sequence is contained therein. Some peptides were identified within 0.5 Da (Daltons) of the predicted value, which was considered to be a sufficient criterion [17].

In the Cape Verde tropical Atlantic waters, there are 52 described *Conus* species, representing about 10% of the worldwide species diversity and only three are non-endemics (*C. ermineus*, *C. genuanus*, and *C. tabidus*. However, this high *Conus* diversity does not exist in other Macaronesian islands [18]. The conopeptides distribution by zoogeographic regions indicates a lack of information regarding the Atlantic Ocean. In this regard, 184 experimentally verified sequences have been reported from the Indo Pacific region and 25 from the Eastern Pacific region [19]. Only seven experimentally verified sequences from the Eastern Atlantic and Mediterranean regions, 22 from the Western Atlantic and Caribbean regions [19], and one conopeptide sequence from South African (with 18 endemic *Conus* species) have yet been reported [5]. In this study, we characterized the conopeptides from the *C. crotchii* venom duct using mass-matching approach (error ≤0.5 Da). *C. crotchii* have a heavy shell, with a greenish ground color, normally with fine spiral dark brown lines. It lives in shallow water (5 meters deep), and was observed only on south of Boa Vista island (Santa Mónica beach). The natural habitat is shown in Figure 1. Usually the *C. crotchii* length is nearly 30 mm. To our knowledge, this is the first description of conopeptides from the venom of Cape Verde endemic *Conus*.

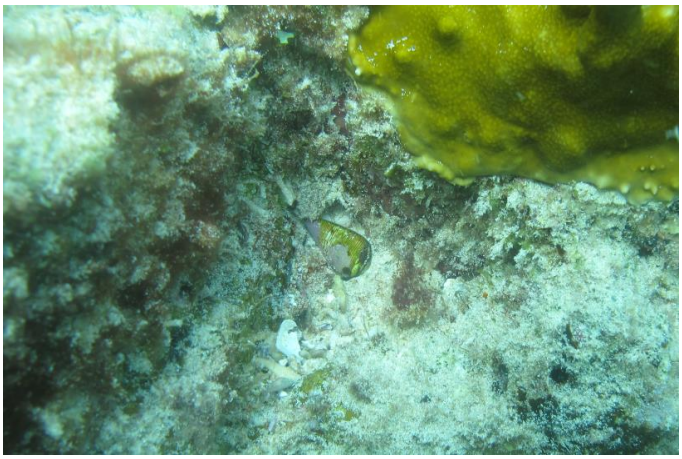

Figure 1: The endemic *C. crotchii* can be found only on Boa Vista Island - Santa Mónica beach.

2. Results and Discussion

2.1. Peptide Mass Range Distribution

The venom sample was first fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2 A, SDS-PAGE only) and 2DE gel (data not shown). Each protein band was isolated and subjected to reduction, alkylation and trypsin digestion. Peptide samples were subsequently submitted to MALDI-TOF MS analysis, enabling the detection of 488 unique molecular masses, ranging from ~700 Da to ~3000 Da. The same technique was used to characterize *C. consors* peptides, rendering the detection of similar number of molecular masses (e.g., 550) [20]. The molecular mass distribution of the conopeptides in *C. crotchii* is shown in Figure 2B. Almost 90% of these masses were between 700 and 1900 Da and only 10% corresponded to large peptides. However, most of the peptides were between 1000 and 1600 Da. The *C. crotchii* molecular mass range is asymmetrically distributed, as described in the case of other cone snail venoms, namely from *C. consors* [20], *C. textile*, *C. imperialis* and *C. marmoreus* [6]. The mass range between 1000 and 2000 Da is the best range MALDI-TOF-MS detection for conopeptide with lower hydrophobicity [16].

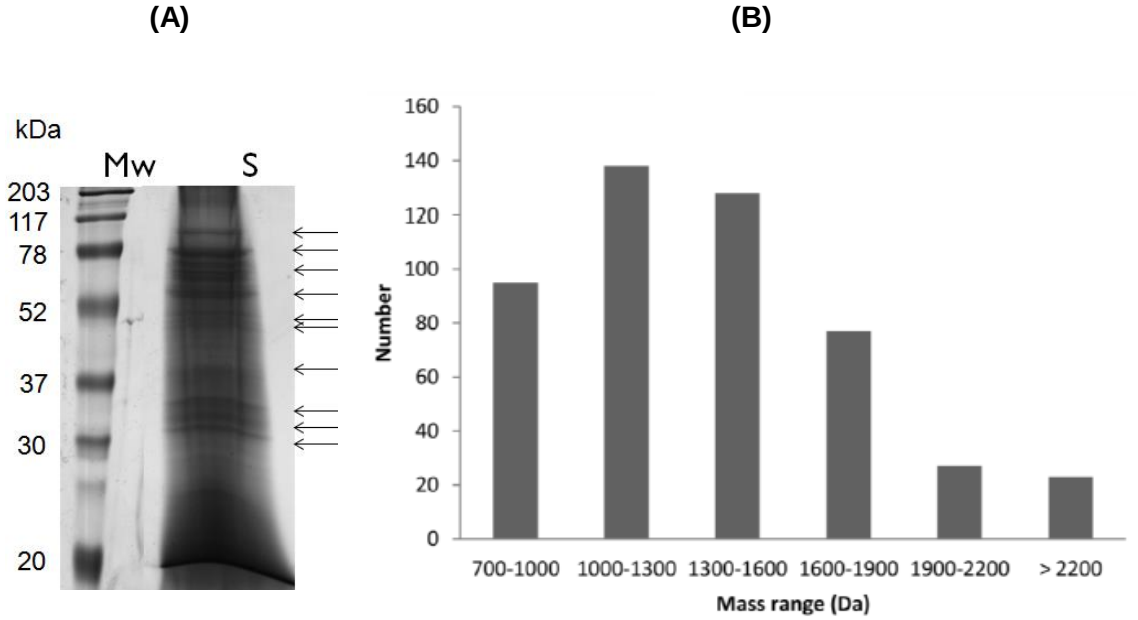

Figure 2: **(A) Proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) from the venom duct of Conus crotchii. Mw: molecular weight, and S: sample; (B) Molecular mass range distribution of the 488 peptides detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis.**

2.2. Peptides Sequence

Conus venom peptides are classified into two groups: the disulfide-poor and the disulfide-rich [21]. The disulfide-rich peptides, also called conotoxins, contain one or more disulphide bridges. In this study, we are able to identify only disulfide-rich peptides by mass-matching (Table 1). These represented only 5% of all "*conomass*" detected. The 95% of conomasses not identified may thus represent an interesting pool of new conopeptides. Databases from National Center for Biotechnology Information (NCBI), UniProtKB, and Conoserver were used to match peptide masses predicted from sequences with a list of masses experimentally obtained in MASCOT search engine tool. This approach can facilitate the validation and accelerate the discovery of conopeptides [22] despite the high frequency and variability of post-translational modifications (PTM) displayed by conopeptides. Extensive PTM are common in *Conus* peptides like as hydroxylation of lysine to 5-hydroxylysine, cyclization of *N*-terminal glutamine to pyroglutamate or amidation of *C*-terminus [23]. Disulfide bonds are the most common PTM in conopeptides and are found in 220 of the 234 conopeptides isolated at the protein level [19].

Table 1: Peptide mass detected by MALDI-TOF-MS and putative sequence by mass-matching using MASCOT database research

Conopeptide	Calculated Mass (Da)	Observed Mass (Da)	Sequence	Cysteine
1	1916.99	1916.90	TKTDDDVPLSSLRDNLK	4
	2042.78	2042.72	ECCEDGWCCTAAPLTGR	
2	795.44	795.48	EQHLIR	3
	2232.96	2233.05	CCDFVKYVGMNPPADKCR	
3	1701.89	1701.80	LWALMKGPRQCTPK	4
	2042.73	2042.69	DAPCDDNNQCCSGLECK	
4	2545.05	2544.99	RPECCSDPRCNSTHPELCGGR	4
	1076.56	1076.57	IRASEGCRK	
5	1158.67	1158.62	AVGLIDKMRR	4
	1786.82	1786.88	KGDRCGTHLCCPGLR	
6	1790.81	1790.87	FQFLNFCCNEK	4
	1693.79	1693.80	ILEDIVSTALATCCK	
7	1687.88	1687.88	ASDGGNAAASDLIALTIK	4
	1970.76	1970.66	G**CC**SRPP**C**ALSNPDY**C**G	
8	798.32	798.29	**C**VGV**C**FG	4
	1790.83	1790.89	EQNKT**CC**GLTNGRPR	
9	2042.76	2042.69	SGGA**C**NSHDQ**CC**INF**C**R	5
	798.34	798.32	KATST**C**M	
10	1169.51	1169.55	NFGDTRS**C**GR	4
	1190.53	1190.55	RGKP**C**P**CC**R	
11	966.49	966.46	K**C**NRFNK	4
	2205.99	2206.08	IPNQK**C**FQHLDD**CC**SRK	
12	985.45	983.46	RGHGRS**C**PG	3
	1352.56	1352.59	NG**C**T**C**VYHWR	

The experimental MS spectra data was used in MASCOT database search tool to retrieve peptide sequences [24]. Here the molecular masses (monoisotopic) of the predicted peptides were compared with the estimated experimentally and the mass error was set as less than 0.1 Da. This high-quality conformity between predicted and observed masses lends confidence to the assignments (error ≤0.5 Da) [25]. To estimate the number of Cys residues in the peptide, disulfide bonds were reduced with dithiothreitol (DTT) and the Cys residues alkylated with iodoacetamide (IAA). This procedure is normally used for identifying disulfide-linked peptides and consequently the number of Cys using MALDI-TOF MS [26–29]. The results suggested the addition of carbamidomethyl group to each sulfur atom and a corresponding increase of 58 Da for each Cys in the peptide. The same chemical modification was suggested for peptide cal12a and cal12b from *C. californicus* [30].

2.3. BLAST Search for Conotoxins

All reduced and alkylated venom peptides from *C. crotchii* were analyzed by MS and amino acid sequence data were suggested by a MASCOT database search and blast analysis in UniProtKB. Peptides identified in the venom by MALDI-TOF MS are underlined (Table 2). Signal, propeptide and mature peptide regions are shown in red, blue and black, respectively (Table 2). For MALDI-TOF MS analysis we used the venom duct and venom gland (see Figure 4) that can likely be related with 33% and 67% propeptide and mature peptide retrieved, respectively (Figure 3). The large percentage (67%) of peptide sequence corresponds to the mature peptide region due to the fact that the sample preparation was done with only the venom duct. Similarly, a total of 12 conotoxins were detected in the *C. crotchii* venom duct, considering only results with a protein 100% max. identity in UniProtKB (Table 2). A BLAST search on UniProtKB database resulted with an *E*-value between 1e-12 and 7e-30 and score bits between 151 and 257. The statistics from BLAST alignments was based on the marginally significant criterion *E*-value of 0.05, with normalized score of ~38 bits [31].

Table 2: Conotoxins identification by BLAST search on UniProtKB database; all 151 results have a 100% Max identity with the protein sequence. Signal, propeptide and mature peptide regions are shown in red, blue and black, respectively. Peptides identified in the venom by MALDI-TOF-MS—MASCOT database are underlined

Conotoxin (Accession)	Protein sequence	*E*-Value [a]
TxVA (P81755.2)	MRCFPVFIILLLLIASAPCFDARTKTDDDVPLSSLRDNLKRTIRTRLNIRE CCEDGWCCTAAPLTGR	2e-12
im23.3 (D0PX86.1)	MIMRMTLTLFVLVVMTAASASGDALTEAKRIPYCGQTGAECYSWCKE QHLIRCCDFVKYVGMNPPADKCR	7e-21
Ec15a (B0KZ79.1)	MEKLTILILVATVLLAIQVLGQGEGEKPPKEWVQQYAAKRLWALMKGP RQCTPKDAPCDDNNQCCSGLECKCFNMPDCQSGSTCRV	3e-28
Ai1.2 (P0CB08.1)	MFTVFLLVVLATTVVSSTSGRRAFRGRNAAAKASGLVGLTDRRPECC SDPRCNSTHPELCGGRR	9e-17
Bu2 (P0CY61.1)	MKLTCVLIIAVLFLTAITADDSRDKQVYRAVGLIDKMRRIRASEGCRKK GDRCGTHLCCPGLRCGSGRAGGACRPPYN	7e-30
Ca5.1 (P0C666.1)	MRCVPVFIILLLLASPAASDPLEKRIQSDLIRAALEDADTKNDPRILEDIV STALATCCKFQFLNFCCNEK	4e-22
PnMGMR-02 (Q9BP56.1)	MGMRMMFTVFLLVVLATTVVSFTSDRASDGGNAAASDLIALTIKGCCS RPPCALSNPDYCG	8e-30
VnMSGL-0123 (Q9BP59.1)	MSGLGIMVLTLLLLVSMATSHQDGGGKQATQRDAINVRRRRSITRRE VVTEE**C**EEYCKEQNKT**CC**GLTNGRPR**C**VGV**C**FG	2e-15
Eb6.18 (C7T1P1.1)	TRSGGA**C**NSHDQ**CC**INF**C**RKATST**C**M	2e-19
Leo-O2 (P0C903.1)	MKLTCVLIIAVLFLTACQLVTADYSGDEQQYRAMRLIDAMRNFGDTRS **C**GRRGKP**C**P**CC**RGFR**C**TGSF**C**RKWQ	1e-12
PVIIA (P56633.2)	VVIVAVLFLTACQLITADDSRRTQKHRALRSTTKLSLSTR**C**RIPNQK**C**F QHLDD**CC**SRK**C**NRFNK**C**V	7e-20
VxXXB (P0C1W6.2)	MPKLAVVLLVLLILPLSYFDAAGGQAVQGDWRGNRLARDLQRGGRD DESE**C**IINTRDSPWGR**CC**RTRM**C**GSM**CC**PRNG**C**T**C**VYHWRRGHGR SC**C**PG	2e-14

[a] The reported *E*-values were derived by the BLAST analysis.

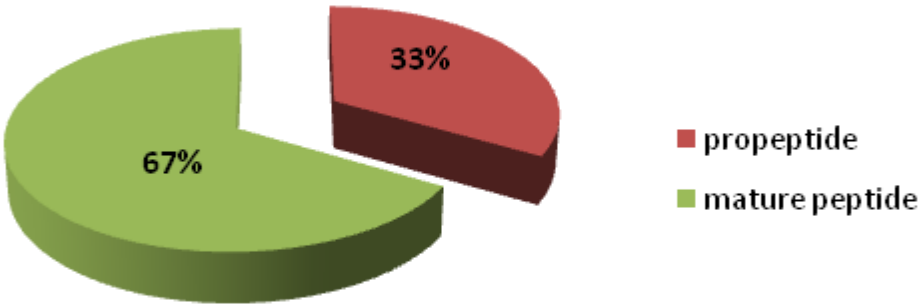

Figure 3: Distribution of peptide sequence identified as "Conoprotein".

All Cape Verde endemic *Conus* species are usually regarded as vermivorous, but it is not entirely clear that they are exclusively vermivorous [18]. The toxin's sequence similarity results indicates that *C. crotchii* venom presents peptides from vermivorous (50%), but also molluscivorous (33%) and piscivorous (17%) *Conus* species. Among them, four conotoxins (Eb6.18, Leo-O2, Bu2, PVIIA) could be assigned to the O1-superfamily, three to the A-superfamily (im23.3, Ai1.2, PnMGMR-02), two to the T-superfamily (Ca5.1, TxVA) and one for each O2- (Ec15a), O3- (VnMSGL-0123) and D-superfamily (VxXXB), all of them previously described (Table 3). These results may suggest that the venom from *C. crotchii* has not only a dietary function but it is used for all kinds of environmental interaction as predators defense. On the other hand, it has already been demonstrated that size and diversity of the conopeptide gene superfamilies on vermivorous species differ significantly [5]. *Conus* peptides from the same superfamily share the typical arrangement of Cys residues in the mature toxin region, the "Cys pattern". Each "Cys pattern" generally corresponds to a precise disulfide framework. However, within the same superfamily, thereissome altered loop spacing of amino acids between cysteines. For example, in the superfamily O, conopeptide μO-MrVIA (*C. marmoreus*) has interval residue number CX6CX9CCX4CX4C (6-9-4-4) and κ-PVIIA (*C. purpurascens*) has CX6CX6CCX3CX5C (6-6-3-5) [32]. Conopeptides from the superfamily O (cysteine framework "C-C-CC-C-C") has a O1, O2 and O3 variation, can blockvoltage-gated Ca^{2+} channels and inhibits voltage-gated K^{+} channels [19]. However, the A-superfamily conopeptides (cysteine framework "CC-C-C"), one of the most studied superfamiles, together with the superfamilies O and T [19], can act on nicotinic acetylcholine receptors (nAChR) and can also block K^{+} channels [33].

Table 3: Conotoxins isolated from different *Conus* species

Name	*C.* species	Diet	Superfamily	Family	Cys pattern (framework)	Reference
Ec15a	*C. emaciatus*	v	O2	unknown	C-C-CC-C-C-C-C (XV)	[31]
Ca5.1	*C. caracteristicus*	v	T	unknown	CC-CC (V)	[34]
im23.3	*C. imperialis*	v	A	unknown	C-C-C-CC-C (XXIII)	[35]
Eb6.18	*C. ebraeus*	v	O1	unknown	C-C-CC-C-C (VI/VII)	[36]
Leo-O2	*C. leopardus*	v	O1	unknown	C-C-CC-C-C (VI/VII)	[37]
VxXXB	*C. vexillum*	v	D	α	C-CC-C-CC-C-C-C (XX)	[38]
TxVA	*C. textile*	m	T	ϵ	CC-CC (V)	[39]
Ai1.2	*C. ammairalis*	m	A	α	CC-C-C (I)	[31]
PnMGMR-02	*C. pennaceus*	m	A	α	CC-C-C (I)	[31]
VnMSGL-0123	*C. ventricosus*	m	O3	unknown	C-C-CC-C-C (VI/VII)	[31]
Bu2	*C. bullatus*	p	O1	unknown	C-C-CC-C-C (VI/VII)	[40]
PVIIA	*C. purpurascens*	p	O1	κ	C-C-CC-C-C (VI/VII)	[41]

v: vermivorous; m: molluscivorous; p: piscivorous.

3. Experimental Section

3.1. Cone Snail Specimen and Venom Extraction

The *Conus crotchii* were obtained from the Boa Vista Island in the Cape Verde archipelago. The venom ducts were dissected on ice and the venom duct removed and diluted in 500 µL of 0.1% formic acidand stored at −80 °C until use. The image was obtained (Figure 4) using magnifying glass Olympus DP72, DF lenses mode (Tokyo, Japan). The venom ducts were mechanically disrupted by ceramic beads (diameter 1.4 mm) using Precellys 24 homogenizer (5400 rpm, 2 × 15 s; Bertin, Montigny-le-Bretonneux,

France). Ceramic beads and insoluble materials were removed by centrifugation at 4 °C (16 × *g* for 10 min, twice). After centrifugation, all venom extract were immediately stored at −20 °C prior to analysis.

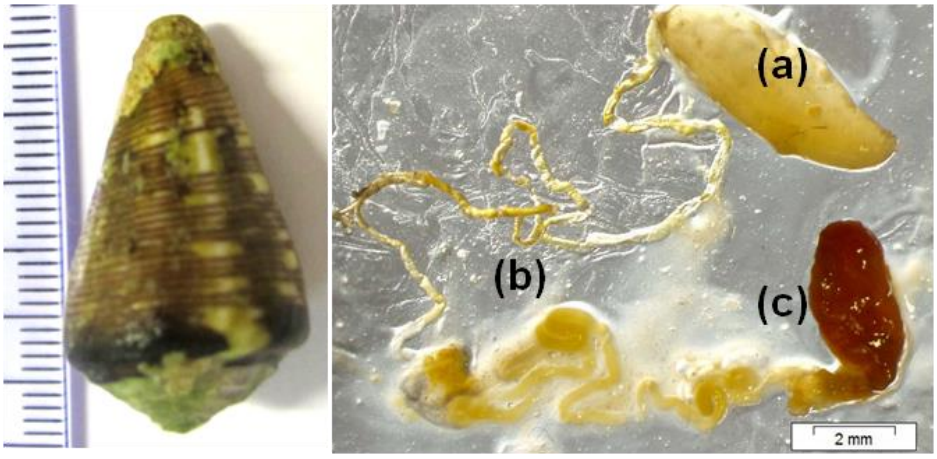

Figure 4: Shell and venom apparatus of *C. crotchii*: (a) Venom bulb; (b) Venom duct; (c) Venom gland.

3.2. Sample Fractionation, SDS-PAGE

Protein samples were mixed with loading buffer with Tris-HCl (0.25 M) pH 6.8, SDS (6%, w/v), glycerol (30%, v/v), β-mercaptoethanol (6.25%, v/v) and proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in homogeneous gels (12% acrylamide) according to Laemmli [42]. Gels were stained with colloidal Coomassie Blue [43], gel images acquired using GS-800 calibrated scanner (Bio-Rad, Hercules, CA, USA) and the protein profiles analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

3.3. Protein Reduction, Alkylation and Trypsin Digestion

Proteins were isolated from the SDS-PAGE gels and submitted to *in-gel* reduction and alkylation to disrupt disulfide bonds [44,45], and thereafter to trypsin digestion. Reduction was achieved by adding 50 µL of DTT (10 mM) prepared in NH_4HCO_3 (100 mM, pH 8.0) to the protein samples followed by incubation during 45 min at 56 °C. For alkylation the dithiothreitol (DTT) was replaced by 50 µL IAA (55 mM) prepared in NH_4HCO_3 (100 mM) and the protein samples incubated during 30 min in the dark. For trypsin digestion dried protein gel bands were incubated with 6.7 ng trypsin/µL during 30 min in ice. Thereafter, the excess of trypsin solution was removed and 5–25 µL of NH_4HCO_3 (50 mM) was added in order to cover the gel pieces. Trypsin digestion

proceeded overnight (15 h) at 37 °C. The solution (supernatant) containing the peptides was subsequently collected into an eppendorf tube and stored at −20 °C.

3.4. MALDI-TOF-MS Analysis

The peptide samples were concentrated and cleaned according to the manufacturer's instructions on a micro C18 ZipTiP column (Millipore, Bedford, MA, USA). The peptides were eluted directly onto the MALDI plate using the matrix α-cyano-4-hydroxycinnamic acid (α-CHCA) at 5 mg/mL prepared in ACN (50%), and formic acid (0.1%). Peptide mass spectrometry analyses were performed by MALDI-TOF/TOF (4700 Proteomics Analyzer, AB SCIEX, Foster City, CA, USA) method described [46–48] in reflector positive mode (700–4000 Da). The experimental mass spectra were searched against the UniprotKB protein sequence database with the Mascot (Matrix-Science, London, UK) algorithm, integrated in the GPS Explorer software (AB SCIEX, Foster City, CA, USA). The search parameters were up to two maximum trypsin missed cleavages, mass tolerance of 50 ppm, cysteine carbamidomethylation (fixed modification), methionine oxidation (variable modification) and a charge state of +1.

4. Conclusions

In this work we characterized the peptide profile from *C. crotchii* using MALDI-TOF and mass-matching. The number of molecular masses studied here resembles the outputs from other studies performed in the genera *Conus* enabling us to validate our approach. We were able to identify several disulfide-rich conotoxins in *C. crotchii* venom duct samples that belong to O1-superfamily (Eb6.18, Leo-O2, Bu2, PVIIA), A-superfamily (im23.3, Ai1.2, PnMGMR-02), T-superfamily (Ca5.1, TxVA) and O2-(Ec15a), O3- (VnMSGL-0123) and D-superfamilies (VxXXB). Taking into consideration the mode of action of some of these conotoxins we may consider that the high diversity of conotoxins may not only be related to diet but with all kinds of environmental interaction as predators' defense. These putative conotoxins may block voltage-gated Ca^{2+} channels, inhibit voltage-gated K^{+} channels and act on nAChRs [15,30]. A large number of masses were not assigned in this work. This promises new research potential and the discovery of new bioactive molecules from Cape Verde *Conus* species.

Acknowledgments

We thank Zinho Évora, Corrine Almeida, Livinho Tavares (Pelé) and Evandro Lopes for providing indispensable field assistance. This work was supported by a PhD grant (SFRH/BD/51477/2011) and was partially funded by the Project MARBIOTECH (reference NORTE-07-0124-FEDER-000047) within the SR & TD Integrated Program MARVALOR—Building research and innovation capacity for improved management and valorization of marine resources, supported by the Programa Operacional Regional do Norte (ON.2—O Novo Norte) and by the European Regional Development Fund, by the project PesT-C/MAR/LA0015/2011 from the Portuguese Foundation for Science and Technology (FCT), INTERREG IV projects ATLANTOX, PHARMATLANTIC, AA was funded by the project PTDC/AAC-AMB/121301/2010 (FCOMP-01-0124-FEDER-019490) from FCT.

Conflicts of Interest

The author declares no conflict of interest.

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Chapter 3

Characterization of a δ-Conotoxin from *Conus ateralbus*, a Vermivorous Cone Snail from the Cape Verde Archipelago

3. Characterization of a δ-Conotoxin from *Conus ateralbus*, a Vermivorous Cone Snail from the Cape Verde Archipelago

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Abstract

Conus ateralbus is an endemic cone snail that has been found only on the west side of the island of Sal, in the Cape Verde Archipelago off West Africa. We describe the collection and first toxinological characterization of this species. An excitatory activity that acted on a majority of mouse lumbar dorsal root ganglion neurons was purified and characterized from *C. ateralbus* venom. We name this 30AA venom peptide, δ-conotoxin AtVIA. An analog of AtVIA with conservative changes on two amino acid residues at the C-terminal region was synthesized and this analog produced an identical effect on the mouse neurons. AtVIA has conserved sequence elements when compared to δ-conotoxins from fish-hunting *Conus* species, and from a peptide purified from *Conus tessulatus*, a species in the worm-hunting Indo-Pacific clade Tessiliconus (Aman, et al. 2015). In contrast, there is no comparable sequence similarity with δ-conotoxins from the venoms of molluscivorous *Conus* species. A rationale for the presence of δ-conotoxins, that are potent in vertebrate systems in two different lineages of worm-hunting cone snails, is discussed.

1. Introduction

The cone snails (genus *Conus*) are a biodiverse lineage of venomous predators; most species specialize in envenomating a narrow range of prey. On the basis of their primary prey, the ~500 species in the genus *Conus* are divided into three broad classes, fish-hunting, snail-hunting and worm-hunting species; the great majority of *Conus* are vermivorous. Pioneering studies on cone snail venoms by Endean and coworkers (Endean and Rudkin 1965) demonstrated that the efficacy of the venom observed on particular animals could be correlated to the prey of that species. Fish-hunting cone snail venoms were highly potent on vertebrates, with worm-hunting *Conus* venoms much less so. Snail-hunting *Conus* venoms are extremely potent when tested on gastropods, but much less effective in vertebrate systems. Thus, the venom components that are highly expressed are presumably under strong selection for high potency and efficacy on molecular targets in the prey of each *Conus* species.

The initial characterization of one family of conotoxins, the δ-conotoxins, followed this general pattern. The first venom peptides of this family identified were from snail-hunting *Conus* species, such as *Conus textile* (Fainzilber, et al. 1995) or *Conus gloriamaris* (Shon, et al. 1994). Although these were highly potent when tested on molluscan systems, they were relatively inactive on vertebrates. In contrast, δ-conotoxins from fish-hunting cones (such as δ-conotoxin PVIA) (Shon, et al. 1995), from *Conus purpurascens*, and δ-conotoxin SVIE (West, et al. 2005), from *Conus striatus*) were extremely potent when tested on fish or mice.

Thus, it was a surprise to discover a δ-conotoxin from a worm-hunting species, *Conus tessulatus* that was highly potent and efficacious on vertebrate systems. Aman et al (2015) rationalized their discovery by suggesting that the ancestral worm-hunting species that gave rise to fish-hunting lineages of *Conus* had evolved a δ-conotoxin which was used for competitive interactions with fish. Every worm-hunting cone snail, as it consumes its worm prey, has a potential problem: teleost fish compete for the same prey. It was suggested that a δ-conotoxin, which would activate Na channels in the pain circuitry, could serve as a powerful deterrent to the fish competitor.

The pre-adaptation to deter fish competitors was postulated to be a critical evolutionary step in the shift to piscivory by cone snails. If a K-channel blocker acting on the same circuitry were subsequently evolved, this would result in a powerful tetanic paralysis of the fish. Major lineages of fish-hunting cone snails have been shown to have both a δ-

conotoxin, as well as a κ-conotoxin that blocks K channels, a combination known as the “lightning-strike cabal”. Thus, the presence of δ-conotoxins in the ancestral worm-hunting *Conus* was postulated to be critical in the shift from worm hunting to fish hunting. If this ancestor of fish-hunting lineages had indeed evolved a δ-conotoxin effective on fish, then related peptides might still be found in worm-hunting lineages descended from the same ancestral species, such as *Conus tessulatus*.

The present phylogenetic organization of the genus *Conus* is shown in Figure 1. Fish-hunting cone snail lineages are shown underlined, and the position of *Conus tessulatus*, the worm-hunting species in the *Tesseliconus* lineage that yielded the δ-conotoxin described above is shown by the red arrow. The phylogeny shown in Figure 1 is consistent with the hypothesis of Aman et al. However, there are a diversity of other worm-hunting lineages that have presumably descended from the same ancestral species, indicated by boxed lineages (mollusc-hunting lineages, marked by checks, are also predicted to have evolved from the same worm-hunting ancestor). In this report we demonstrate that one of the other descendant worm-hunting lineages, which is distant from *Tesseliconus* (the lineage that gave rise to *Conus tessulatus*), and which is presently restricted to an entirely different biogeographic range, does indeed contain a δ-conotoxin that is highly potent on vertebrate targets. This peptide was discovered in a lineage of West African cone snails, the subgenus *Kalloconus*.

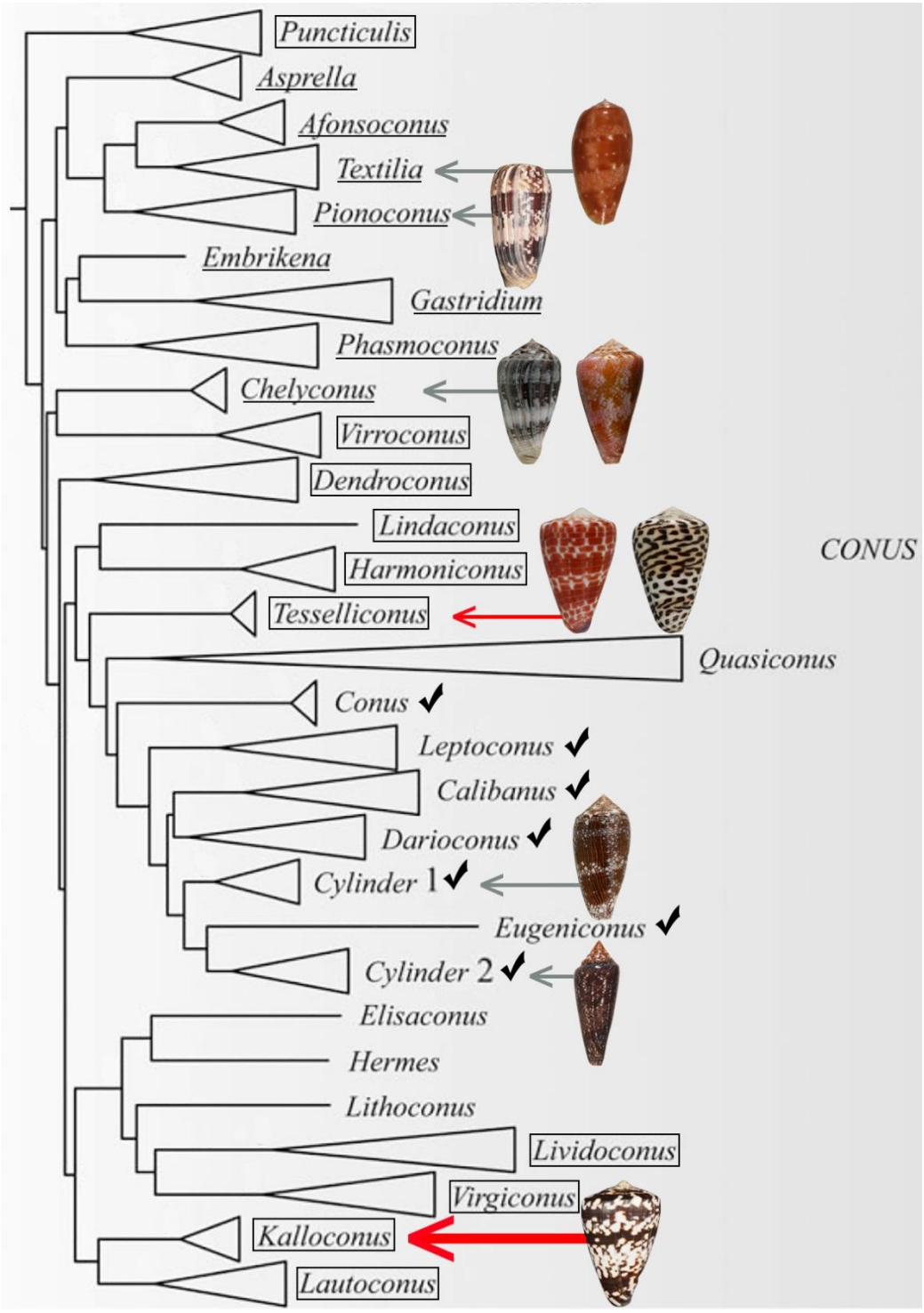

Figure 1: A phylogenetic tree showing the large clade of *Conus* encompassing all lineages that are fish hunting (underlined) and snail hunting (checked). Well established worm-hunting lineages are boxed. δ-conotoxins have been characterized from the 9 species figured, including the worm-hunting *Conus tessulatus* (red arrow). In this work, we investigated the venom of *Conus ateralbus* (shown by the thick red arrow), which is in the Kalloconus lineage. This tree was adapted from a figure from Puillandre et al. (2014). Species discussed in this study whose shells are figured are (from top to bottom): *Conus bullatus* (Textilia); *Conus striatus* (Pionoconus); *Conus ermineus* (l) and *Conus purpurascens* (r) (Chelyconus); *Conus tessulatus* (l) and *Conus eburneus* (r) (*Tesseliconus*); *Conus textile* and *Conus gloriamaris* (Cylinder); *Conus ateralbus* (*Kalloconus*).

A phylogenetic tree showing *Conus ateralbus*, the species analyzed and other species in the subgenus is shown in Figure 2. This is the first venom characterized from any species in the *Kalloconus* lineage. The subgenus *Kalloconus* is restricted to tropical West Africa, from the Islands of Madeira, to South Angola (in contrast, *Tesseliconus* is only found in the Indo-Pacific). *Kalloconus* species comprise some of the larger *Conus* species found in the Eastern Atlantic, including *Conus pulcher*, the largest species in the entire superfamily Conoidae, growing to a length of 230mm. Although the shells of *Kalloconus* species have long been used as cultural objects in northwest Africa, as well as being prized collector's items for many centuries, little was known about their biology, and some of the species in this clade have only recently been described. The focus of this article is a single species of *Kalloconus*, *Conus ateralbus*, and a specific venom component from this species that has broader significance for toxinology.

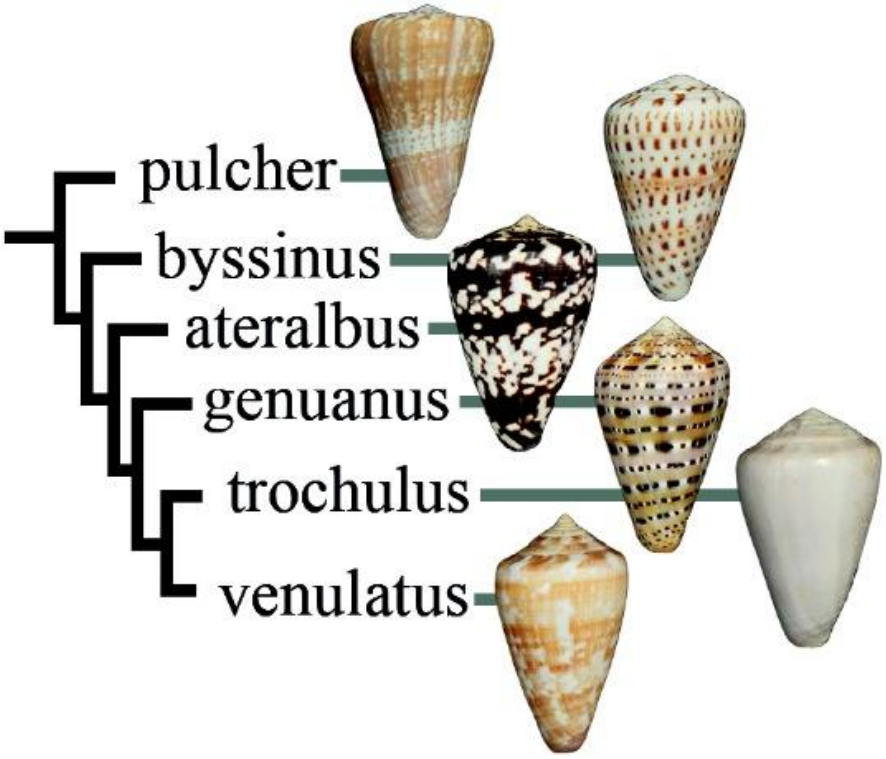

Figure 2: Relationship of species in *Kalloconus*. Most of the species are approximately the same size (ca 40 mm), except for *Conus pulcher*, which can be very much larger (up to 230 mm).

This is the first toxinological study on any species in the subgenus *Kalloconus*. We detail both the collection data and the biological observations made in the field regarding *Conus ateralbus*; some of these may apply more broadly to all *Kalloconus* species. In contrast to some other species in *Kalloconus* (e.g., *Conus pulcher* and *Conus genuanus*) that are widely distributed across the West African marine

biogeographic province, *Conus ateralbus* is an endemic species in *Kalloconus* with the narrowest known biogeographical range. It is restricted to the west coast of the Island of Sal, in the Cape Verde archipelago. It has been suggested that an ancestral *Kalloconus* from the West African coast colonized the Cape Verde archipelago relatively recently (4.6 MYA), giving rise to at least five extant species that are endemic to Cape Verde (Cunha, et al. 2005). The specimens analyzed in this study were collected by the senior author, who also recorded field observations that provide direct evidence for the vermivory of *Conus ateralbus* (and by implication, the entire *Kalloconus* clade).

2. Materials and Methods

2.1. Field collection and venom extraction

Conus ateralbus specimens were collected in the Calheta Funda Bay, Sal Island, in shallow water (around 2 meters deep) in 2013. The specimens were collected in one day, kept alive in seawater and preserved at -20ºC at the end of the day. The venom duct was dissected from each frozen specimen. Venom was obtained from ducts immediately after dissection by placing each duct on an ice-cold metal spatula; the venom was squeezed out using an eppendorf pipette tip and was lyophilized. Crude venom extracts were prepared using 40% (v/v) CH_3CN/water acidified with 0.1% (v/v) TFA.

2.2. Venom fractionation

Crude extract from 36.5 mg of venom was fractionated by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) using a C_{18} Vydac 218TP101522 preparative column. Elution was done at a flow rate of 7 mL/min and a gradient ranging from 10% to 30% of solvent B in 20 min, 30% to 50% in 25 min, 50% to 100% in 30 min, and 100% for 15 min. Solvent B was 90% (v/v) CH_3CN in 0.1% (v/v) aqueous TFA, and solvent A was 0.1% (v/v) TFA in water. The subfractionation of the active fraction 38 was done by RP-HPLC using a C_{18} Vydac monomeric 238EV54 column. The absorbance was monitored at 220 and 280 nm.

2.3. Mass spectrometry and sequence determination

The crude HPLC fractions were analyzed by W. Low using Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectrometry at the Mass Spectrometry Core, Salk Institute for Biological Studies, La Jolla, CA.

The sample (subfraction 38.6, Figure 4B) was dissolved in 100 μL of 0.5% acetic acid. Ten microliters were desalted using POROS R2 beads (Cotto-Rios, et al. 2012). An aliquot of the unreduced sample was loaded onto a 0.2 x 25cm Pepswift EasySpray column. The sample was eluted at a flow rate of 1μL/min with a gradient of 0-100%B (90% (v/v) CH_3CN in 0.5% acetic acid) in 20min, a spray voltage of 2.5kV on an Easy nLC-1000 nanoUHPLC coupled to an Orbitrap Elite mass spectrometer. MS1 scans were acquired at 120,000 resolution (@ 400 m/z). For MS2 the most abundant precursor was isolated and fragmented using ETD at 15,000 resolution (@ 400 m/z) and 60 msec ion ion reaction time.

Another aliquot of the sample was dried in a speedvac, and subsequently reduced and alkylated in vapor using 50% CH_3CN, 1% 2-methylaziridine and 2% trimethylphosphine in 100 mM ammonium bicarbonate pH 8.4) (v/v), for 90 min at room temperature. Alkylation vapor was removed and sample was dissolved in 10 μL of 0.5% acetic acid. Aliquots were analyzed on LC-MS as above, but the gradient was changed to 0-50%B in 50 min. MS1 scans were acquired at 120,000 resolution (@ 400 m/z). MS2 was acquired on the top 5 precursors that carry at least 4 charges using the following settings: 4 microscans, 3 m/z isolation window, target value of 1e4 ions. Each precursor was subjected to ETD and HCD fragmentation using the following conditions: 15,000 resolution (@ 400 m/ z), 30 sec dynamic exclusion, ETD using 60ms ion ion reaction time with supplemental activation, HCD using 27% normalized collision energy. The measured mass deviates from the theoretical mass by 5.5 ppm and is within the mass error of the instrument.

The sequence was obtained by manual de novo sequencing.

2.4. *In vivo* assay

Each dried aliquot of HPLC fraction pools or individual fractions was re-suspended in 12 μL of normal saline solution (NSS, 0.9% NaCl). Mice (15 days old, 6-8 g of body weight) were intra-cranially injected using a 0.3 ml insulin syringe; the same volume of NSS alone was injected in control mice. After the injection, the peptide-injected mice

were observed side by side with NSS-injected controls, all placed in separate cages for at least 1 h (Olivera, et al. 1999).

2.5. Calcium imaging assay on DRG

Dorsal root ganglia (DRG) were dissected following previously described protocols (Teichert, et al. 2012). Lumbar DRG neurons from wild type C57/BL6 mouse were dissociated, pooled and cultured overnight for calcium imaging experiments. Cells were loaded with Fura-2-AM dye one hour before the experiment. During the experiment, the dye inside the cells was excited alternately with 340 nm and 380 nm light. The ratio of the emission 510 nm from both excitations was measured. The ratio of the fluorescence intensity is a measure of the increase in intracellular calcium resulting from the depolarization caused by the external application of 25 mM KCl every seven minutes. After the third KCl pulse, venom extract or HPLC fractions of the venom extract were applied and the effects on the levels of intracellular calcium were monitored. Experimental protocols involving live animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah.

2.6. Peptide synthesis

Based on the early sequencing results, AtVIA[V28L;T30S] peptide with the following sequence: ZCGADGQFCFLPGLGLNCCSGLCLLVCLPS^ was synthesized at 50 µmol scale using an AAPPTec Apex 396 synthesizer (AAPPTec, LLC, Louisville, KY) using standard solid-phase Fmoc (9-fluorenylmethyloxycarbonyl) protocols. Fmoc-protected amino acids were purchased from AAPPTec. Peptides were assembled on pre-loaded Fmoc-L-Ser(tBu)-Wang resin (substitution*, 0.53* mmol·g^{-1}; Peptides International Inc.). Side-chain protection for each corresponding amino acid was: Asp, O-*tert*-butyl (OtBu); Ser, *tert*-butyl (tBu); and Asn, Gln and Cys, trytl (Trt). Coupling of each amino acid was achieved using 1 equivalent of 0.4 M benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 2 equivalents of 2 M *N*,*N*-diisopropylethyl amine (DIPEA) in *N*-methyl-2-pyrrolidone (NMP). The amino acid amounts used were at ten-fold excess (60 min coupling). Fmoc-protecting groups were removed by a 20-min treatment with 20% (v/v) piperidine in dimethylformamide (DMF).

2.7. At6A[V28L;T30S] cleavage, derivatization and purification

Peptide was cleaved from 100 mg resin by treatment with Reagent K (TFA/H_2O/phenol/thioanisole/1,2-ethanedithiol; 82.5/5/5/5/2.5 by volume). After 2.5 h, the crude peptide was separated from the resin by vacuum-filtration. The cleavage product was precipitated in cold methyl-*tert*-butyl ether (MTBE) and subsequently washed one more time with MTBE. Crude peptide was suspended in 50% (v/v) CH_3CN in 0.01% aqueous TFA and treated with 55 mg of [2-(Trimethylammonium)ethyl] methane thiosulfonate bromide (MTSET). After 30 min the pellet was still present in the solution, so another portion of MTSET was added (~40 mg) and it was allowed to react for an additional 1h. The modified peptide was then purified by reversed-phase (RP) HPLC in a Vydac C18 semi-preparative column (218TP510, 250 mm x 10 mm, 5 µm particle size) over a linear gradient ranging from 20% to 50% of solvent B in 30 min with a flow rate 4 mL/min. The peptide was quantified by analytical RP- HPLC comparing the area under the peak to the area of a known amount of the a reference peptide At6A[F8Y;V28L;T30S]. Out of 100 mg cleaved resin, ~500 nmol of thiocholine-modified linear peptide was obtained. The identity of the peptide was confirmed using ESI MS: calculated:$[M^+]$= 3721.75, obtained:$[M^+]$= 3717.67.

2.8. Oxidative folding of At6A[V28L;T30S]

The linear, thiocholine-modified peptide (100 nmol) was re-suspended in 50% (v/v) CH_3CN in 0.01% aqueous TFA and added to a mixture of 4 mL 0.2 M Tris-HCl +2mM EDTA, pH=8.7, 0.4 uL (5%) Tween 40, 1.6 mL 1:1mixture of 10 mM GSSG and 10 mM GSH and 1.5 mL water. Folding reaction was conducted for 4h at room temperature and quenched by acidification using 8% (v/v) formic acid. Peptide was purified by RP-HPLC on the C18 semipreparative column using two different gradients: 35% to 95% change of solvent B in 15 min (3%/min of gradient change, 4mL/min of elution rate) and 35% to 95% change of solvent B in 30 min (2%/min; 4 mL/min). Identity of the peptide was confirmed by MALDI-TOF mass analysis. calculated $[M+H]^+$: 3008.29 Da, observed: $[M+H]^+$: 3008.33 Da, but the desired mass was represented by a minor peak. The major peaks observed were: 3030.33 Da and 3046.30 Da which correspond to: $[M+Na]^+$ (calculated: 3030.27 Da) and $[M+K]^+$(calculated: 3046.25 Da) respectively. There were also masses ranging from 1300 to 1500 Da, indicating traces of Tween (used in the folding reaction) in the sample. The peptide was quantified using amino

acid analysis. Out of 1200 nmols of the linear peptide 174 nmols of the desired folded peptide was obtained.

3. Results

3.1. Collection of *Conus ateralbus*; venom fractionation

Specimens of *Conus ateralbus* can be reliably collected on the west coast of the Island of Sal from April to June; the field collection protocol are detailed under Methods. One specimen of *Conus ateralbus* was found consuming a polychaete worm (see Figure 3). The specimen did not bury itself as it consumed the worm, and it took about one hour — it was thus exposed the entire time it was feeding on its prey. As can be seen in the figure (and in the video supplied as supplemental material), the prey was far longer than the cone snail itself.

Figure 3: ***Conus ateralbus*** **observed in the field feeding on a long worm, many times the length of the predatory snail.**

Conus ateralbus venom from many specimens was pooled and the venom extract was assayed as described under Methods. Both the activity on dorsal root ganglion (DRG) neurons and that upon IC injection into mice were assessed. The venom extract was fractionated; the HPLC chromatogram is shown in Figure 4A. Several biologically-active fractions were detected; one of these, Fraction 38, was further subfractionated and the biologically-active component was purified to homogeneity. The activities on DRG and mice were found in the major peak (Figure 4B); MALDI-MS (linear mode)

revealed this major peak to be homogeneous with a molecular mass of 3010 Da (Figure 4C). In order to determine the number of cysteine residues, the peptide was reduced with DTT and alkylated with 4-vinylpyridine; the mass increment upon pyridylethylation corresponded to the presence of six cysteine residues.

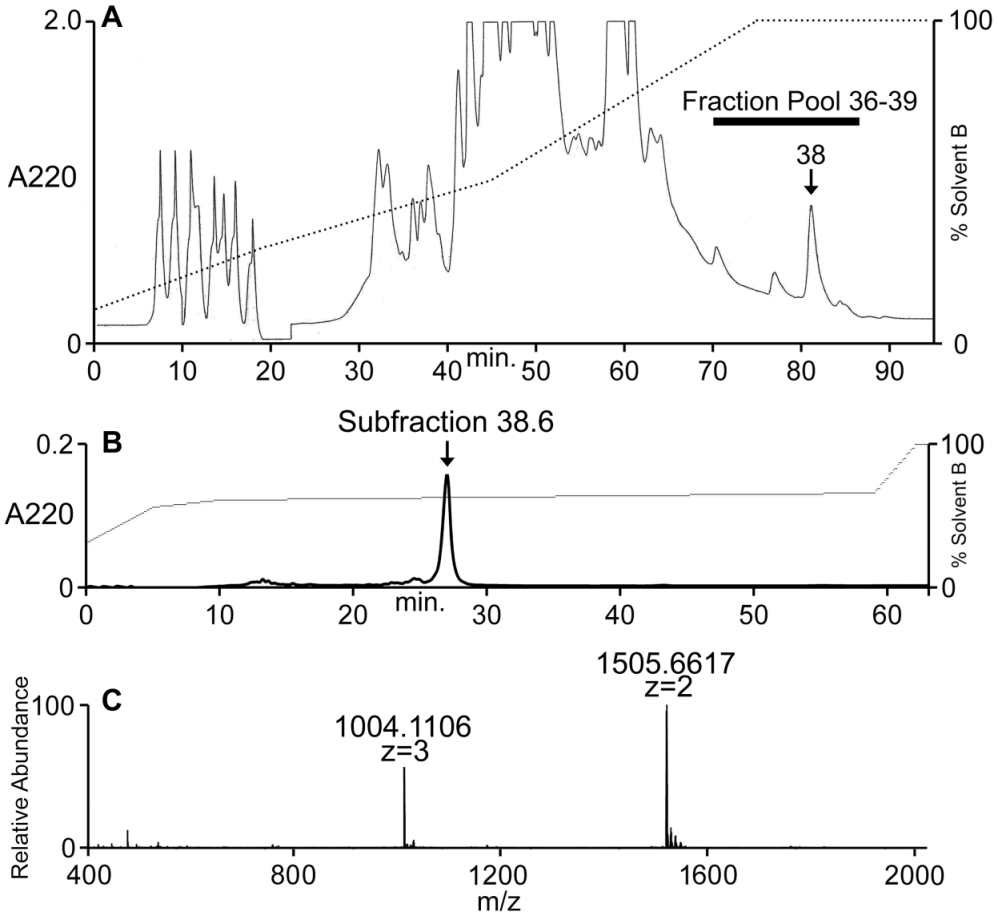

Figure 4: Fractionation of *Conus ateralbus* venom. The peptide characterized in this study was first detected in the pool of fractions shown, 36-39 (Fig. 4A). Further purification of the active fraction (38) yielded the chromatogram shown in Figure 4B. The major peak (subfraction 38.6) was both biologically active and gave the results shown in Figure 4C upon analysis on an Orbitrap Elite mass spectrometer at 120,000 resolution.

3.2. Biological activity

The HPLC fractions were assayed using calcium imaging of native DRG neurons (Teichert, et al. 2012; Imperial, et al. 2014; Aman, et al. 2015) and intracranial injections on mice. The activity that caused excitatory effects on a majority of the dorsal root ganglion cells eluted extremely late (fraction 38), as shown in Figure 4A, suggesting the highly hydrophobic nature of the active component. The behavioral

phenotype elicited by fraction 38 in mice included hypersensitivity to stimuli like touch (Chen, et al. 2008), a response not observed in control mice.

The excitatory activity that was purified and characterized affected >80% of dorsal root ganglion neurons. Some of the results with the purified peptide are shown in Figure 5. In the experiment shown, the cells were exposed to 25 mM KCl, eliciting an increase in cytosolic [Ca^{++}]. If the cells were preincubated with the purified peptide, a large fraction of the cells responded with an increase in the Ca^{++} influx observed after the KCl pulse (first three traces in Figure 5). A small fraction of the cells responded with an increase in cytosolic [Ca^{++}] even without application of KCl (4th trace, Figure 5), and a minor fraction (~15%) of the cells did not respond to preincubation with the peptide (5th trace, Figure 5). It should be noted that the first three traces show cells of different sizes, and the responses to the peptide differed in detail in these individual cells.

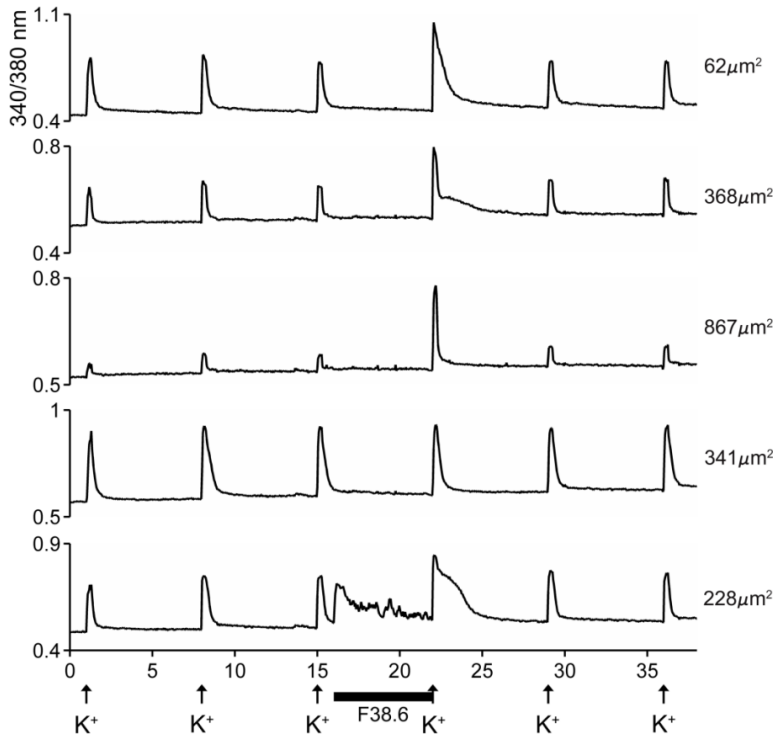

Figure 5: **Responses of 5 different cells to the purified at6a peptide (Fraction 38.6). Shown are the responses of 5 DRG neurons (see Methods); the size of each cell is indicated. A pulse of 25mM KCl was applied as described under Methods; the horizontal bar indicates when cells were incubated with the purified peptide. The first three cells (from top) show a change in response upon depolarization with KCl. The fourth trace shows a cell that directly increased cytosolic Ca^{++} when the peptide was added, even without KCl depolarization (approximately 26% of cells responded in this manner) and the bottom trace shows a cell that did not respond (approximately 15% of DRG neurons were non-responsive to the peptide).**

These results are consistent with the activity of a δ-conotoxin that inhibits the inactivation of voltage-gated Ca channels (see Aman, et al. 2015).

3.3. Sequence determination

The amino acid sequence of the peptide was determined as described under Methods and is shown in Figure 6. The peptide spectrum is consistent with a 30-amino acid peptide with the following sequence: Z**C**GADGQF**C**FLPGLGLN**CC**SGL**C**LLV**C**VPT (where Z is pyroglutamate). It is not possible to differentiate between the isobaric amino acids isoleucine and leucine by mass spectrometry alone and this ambiguity is indicated by (I/L).

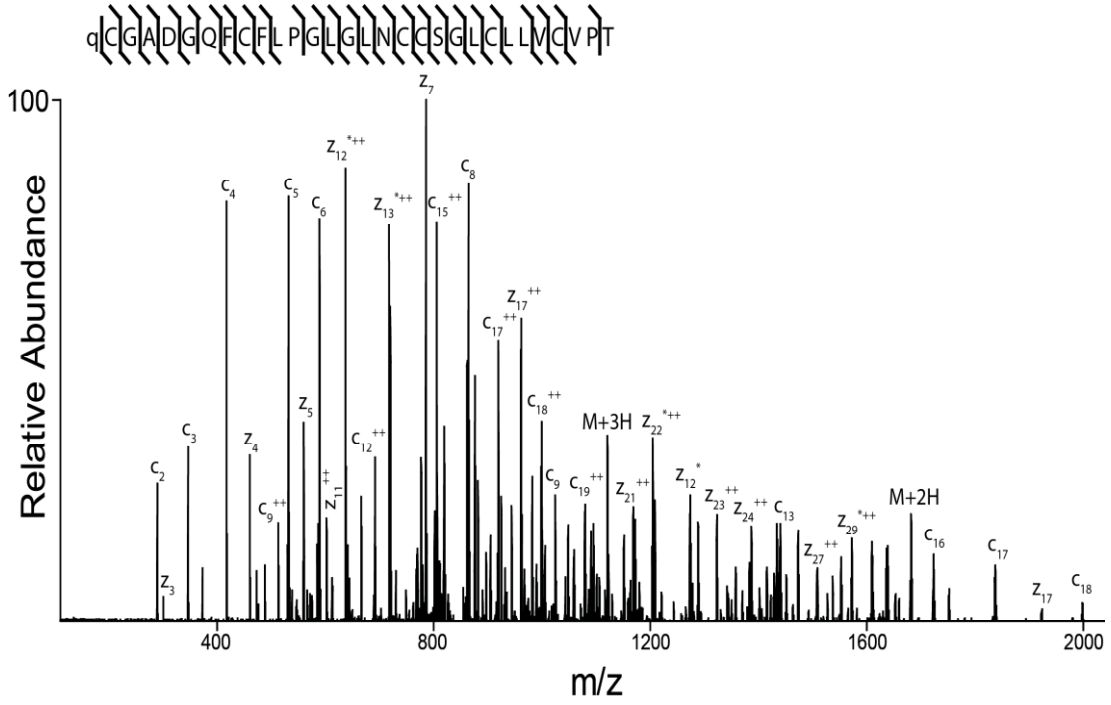

Figure 6: Determination of the sequence of AtVIA by tandem mass spectrometry. MS/MS ETD spectrum of the $(M + 5H)^{+5}$ ion of qCGADGQFCF(L/I)PG(L/I)G(L/I)NCCSG(L/I)C(L/I) (L/I)VCVPT after reduction and alkylation with 2-methylaziridine acquired on the Orbitrap Elite with 15,000 resolution (@ 400 m/z). N-terminal fragment ions (c-type ions) are indicated by ⌉ and C-terminal fragment ions (z-type ions) are indicated by ⌊. Doubly charged ions are indicated with $^{++}$ and z ions resulting from cleavage at cysteine and loss of the cysteine side chain are indicated with *. $[M+5H]^{+++++\bullet\bullet}$ and $[M+5H]^{+++++\bullet\bullet\bullet}$ indicates quintuple charged precursor ions that captured 2 or 3 electrons, respectively, but have not dissociated in fragment ions. Due to space limitations, different charge states of already labeled peptide bond cleavages are not all indicated in the figure. The mass accuracy for all fragment ions is better than 15 ppm. The mass spectrometer used cannot differentiate between isoleucine or leucine and for simplicity leucine is used in the figure to indicate a fragment ion of mass 113.08406.

The arrangement of the six cysteines suggests that this new peptide has an ICK structural motif and belongs to the O-superfamily, with Framework VI (C-C-CC-C-C). Thus, the purified peptide was initially designated as at6a. As shown in Table 1, at6a shares significant sequence homology with other δ-conotoxins, e.g.: δ-TsVIA, *C. tessulatus*; δ-ErVIA, *C. eburneus;* δ-EVIA, *C. ermineus; and* δ-SVIE, *C. striatus*. Together, the activity in DRG neurons and the sequence homology to δ-conotoxin shown in Table 1 are consistent with the peptide being a δ-conotoxin, i.e., inhibiting the inactivation of voltage-gated Na channels. We therefore designate the peptide δ-conotoxin AtVIA.

Table 1: Comparison of δ-conotoxin sequences from various cone snail species

Subgenus (Prey)	*Conus* species	δ-Conotoxin	Sequence		Ref
Kalloconus (Worm)	*C. ateralbus*	δ-AtVIA	Z**C**GADGQF**C**FL-PGLGLN**CC**SGL**C**L-LV**C**VPT	30	This work
Tesseliconus (Worm)	*C. tessulatus*	δ-TsVIA	**C**AAFGSF**C**GL-PGL-VD**CC**SGR**C**F-IV**C**LL	27	Aman *et al*.,2015
	C. eburneus	δ-ErVIA	**C**AGIGSF**C**GL-PGL-VD**CC**SGR**C**F-IV**C**LP	27	Aman *et al*.,2015
Chelyconus (Fish)	*C. purpurascens*	δ-PVIA	EA**C**YAOGTF**C**GIKOGL---**CC**SEF**C**LPGV**C**FG	29	Shon *et al*.,1995
	C. ermineus	δ-EVIA	EA**C**YPOGTF**C**GIKOGL---**CC**SEL**C**LPAV**C**VG	29	Barbier *et al.,* 2004
Pionoconus (Fish)	*C. striatus*	δ-SVIE	DG**C**SSGGTF**C**GIHOGL---**CC**SEF**C**F-LW**C**ITFID	31	West *et al*., 2005
Textilia (Fish)	*C. bullatus*	δ-BuVIA	DE**C**SAOGAF**C**LIROGL---**CC**SEF**C**F-FA**C**F	27	Bulaj *et al*., 2001
Cylinder (Snail)	*C. textile*	δ-TxVIA	W**C**KQSGEM**C**NLLDQN---**CC**DGY**C**IVLV**C**T	27	Hasson *et al*., 1993
	C. gloriamaris	δ-GmVIA	VKP**C**RKEGQL**C**DPIFQN---**CC**RGWN**C**VLF**C**V	29	Shon *et al*., 1994

3.4. Synthesis and folding of AtVIA[V28L;T30S]

The δ-conotoxin family is known to be difficult to synthesize and correctly fold due to their highly hydrophobic character (Bulaj, *et al.* 2001; DeLa Cruz, et al. 2003). While some of them are relatively easy to handle in linear form (e.g. PVIA), others are not.; AtVIA[V28L;T30S] is a good example of such behavior. When suspended in HPLC solvent with high acetonitrile content (>50%) and injected on the C18 column, no peptide peak was observed (Figure 7, Panel A). But when treated with MTSET for 1h 30 min, a major product appeared (Figure 7, Panel B). Its identity was confirmed by mass spec analysis. Methanethiosulfonate reagents (MTS-R) are known to rapidly and selectively react with cysteine residues forming mixed disulfides (Kenyon et al. 1977). All 6 cysteines of AtVIA[V28L;T30S] were modified with thiocholine residues, increasing overall solubility of the peptide, and making it easy to purify by RP-HPLC. Such mixed disulfides are reversible and do not interfere with the subsequent oxidative folding reaction, which was shown before (Luo et al., 2012) for the synthesis and folding of hepcidin via S-sulfonation. Linear AtVIA[V28L;T30S] with thiocholine-modified cysteine residues folded within 4 h in a buffered solution (pH=8.7), in the presence of 5% Tween 40 and 1:1 mixture of reduced and oxidized gluthatione with 14 % yield (Figure 7, Panel C). The temporary peptide modification with MTS-R reagent can be a useful method for improving the solubility of highly hydrophobic conopeptides, in addition to already existing methods, including recently published approach utilizing an acid-cleavable solubility tag (Peigneur et al. 2014).

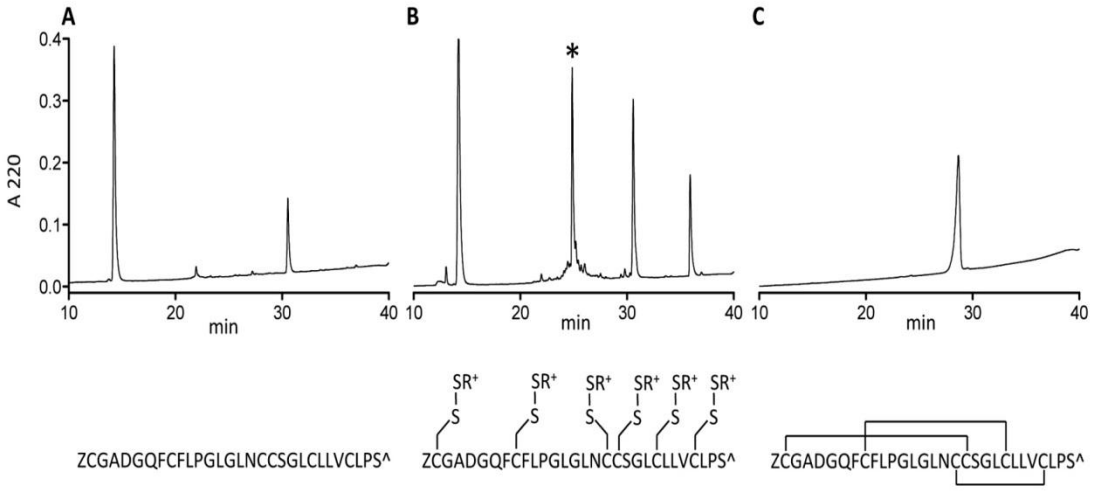

Figure 7: Synthesis of At6A[V28L;T30S]. (A) Crude pellet of At6A[V28L;T30S] was suspended in high acetonitrile content HPLC buffer and injected on C18 RP-HPLC. No peak of desired peptide was

observed. (B) MTS-ET treatment of the crude peptide led to a S-thiocholine modified peptide (as shown below the HPLC chromatogram); the peak of the temporary S-modified peptide is indicated with an asterisk (*) on the HPLC chromatogram. (C) HPLC profile of the folded and purified At6A[V28L;T30S], with an assumed disulfide bond pattern indicated below the HPLC chromatogram.

3.5. Biological activity of AtVIA[V28L;T30S]

The data obtained by calcium imaging in native DRG neurons in the presence of the AtVIA analog is shown in Figure 7. Based on the type and ratio of DRG cells affected, there is no apparent difference between the effect of the native sample of AtVIA (Figure 5) and that of AtVIA[V28L;T30S].

4. Discussion

We report the first toxinological characterization of *C. ateralbus*, an endemic cone snail species from the Cape Verde Islands, an isolated archipelago in the tropical Atlantic Ocean. *C. ateralbus* belongs to the subgenus *Kalloconus*, and is only found on Sal Island (Calheta Funda and Mordeira Bays). While *C. ateralbus* is restricted to Sal, the phylogenetic tree in Figure 2shows a close relationship with two other species endemic to the Cape Verde archipelago, *C. trochulus* (found only in Boa Vista) and *C. venulatus* (found on Sal, Boa Vista, Maio and Santiago) (Duda and Kohn 2005; Cunha, et al. 2008), and with *C. genuanus* (non-endemic). It was previously suggested that *C. ateralbus* was worm hunting, based on the analysis of the radular teeth (Cunha, et al. 2008); we provide direct observations that *C. ateralbus* is indeed a worm hunter (Figure 3). Thus, it is likely that all *Kalloconus* are worm hunting, since specific clades in the genus *Conus* generally sort out on the basis of their primary prey. It is notable that in the phylogenetic tree in Figure 1, there are clusters of worm-hunting lineages that are well separated from each other. *Conus tessulatus*, in the subgenus *Tesseliconus* clusters with *Harmoniconus* and *Lindaconus*. In contrast, *Kalloconus* clusters with *Lautoconus* (also West African) and less closely with *Virgiconus* and *Lividoconus* (both Indo-Pacific).

In the present work, we establish the presence of δ-conotoxins that act on vertebrate Na channels in the two divergent clusters of worm-hunting clades. These δ-conotoxins have strong sequence similarities; the *C. ateralbus* venom peptide, δ-conotoxin AtVIA (δ-AtVIA), was purified to homogeneity and biochemically characterized as detailed above. Table I shows δ-conotoxin sequences from various subgenera of *Conus*,

including fish-hunting, snail-hunting and worm-hunting lineages. As shown in the Table, there are conserved sequence features in venom peptides (highlighted in yellow) from the two worm-hunting and three fish-hunting subgenera identified that are not shared by the peptides from snail-hunting species. The conserved sequence features in fish-hunting and worm-hunting cone snails are presumably important for targeting these peptides to vertebrate Na channels. In contrast, the sequences of δ-conotoxins from snail-hunting species do not share these consensus sequence features and are not effective on vertebrate Na channels.

It should be noted that although there are conserved sequence features between δ-conotoxins from worm-hunting and fish-hunting species, there are also generic differences between the two groups of peptides. For example, the worm-hunting peptides can be separated from those from fish-hunting cone snail venoms by examining the loop between the second and third Cys residues in these peptides. In all the δ-conotoxins from fish-hunting cones, the third amino acid in this loop is always a positively charged residue (i.e., K in the *Chelyconus* sequences, H in the *Pionoconus* peptide and R in the *Textilia* peptide); this positively charged residue is missing from the three peptides from worm-hunting *Conus*. Furthermore, although the proline residue is conserved in this loop, it is posttranslationally modified to hydroxyproline in all of the sequences from fish-hunting species, but is unmodified in the three sequences from worm-hunting species. There are additional generic differences as well. The differences between the peptides derived from fish-hunting versus worm-hunting *Conus* suggest that the spectrum of voltage gated sodium channels might systematically differ between the two classes of peptides. If this were the case, then δ-conotoxins such as the peptide we have characterized here from *Conus ateralbus* may prove to be useful pharmacological reagents for differentiating between the various molecular subtypes of voltage gated sodium channels.

The sequence conservation that is highlighted in Table I is consistent with the hypothesis that the last common ancestor of the four fish-hunting species, whose shells are shown in the figure (i.e., *C. purpurascens*, *C. ermineus*, *C. striatus*, and *C. bullatus*) and the three worm-hunting species (i.e., *C. eburneus*, *C. tessulatus* and *C. ateralbus*) had already evolved a δ-conotoxin with these consensus sequence features as predicted by the hypothesis detailed by Aman et al., (2015) for the molecular events that accompanied the prey shift from worm hunting to fish hunting. However, as shown in Table I, two snail-hunting δ-conotoxins from the subgenus *Cylinder*, δ-TxVIA and δ-GmVIA do not share these consensus sequence features. However, these snail-

hunting species are also descended from the last common ancestor referred to above — this would therefore appear to be inconsistent with the hypothesis of Imperial et al., and Aman et al.

There are two possible explanations for the observed lack of conservation in δ-conotoxins from snail-hunting *Conus* species. First, δ-conotoxins play a major role in prey capture; a snail hunter has the biological problem of keeping the envenomated snail outside its shell. A predator striking a snail would elicit the automatic response of withdrawing deeply into the shell; cone snails have no way of breaking the shell of their prey. The δ-conotoxins are key to activating motor circuitry so that immediately after envenomation, the prey is extended and has spastic irregular movements outside the shell, and is unable to withdraw into its shell (Olivera, et al. 2015 (in review)). This then allows further injection of venom, providing continuous access to the soft parts of the prey without having to break the shell. Thus, the δ-conotoxins that have been purified and characterized undoubtedly play a role in prey capture, so it is not surprising that these are targeted to molluscan sodium channels and would be expected to diverge in sequence from the ancestral δ-conotoxin. Additionally, because the prey of snail-hunting cones have a hard shell, retention of a peptide that deters fish competitors may no longer be required. Thus, these explanations rationalize why δ-conotoxin sequences from *C. textile* and *C. gloriamaris* do not have the consensus features of the other δ-conotoxins in Table I, and why these are not active on vertebrate voltage-gated sodium channels.

Acknowledgements

This work was supported by grants to BMO from the National Institute of General Medical Science, GM 48677 and GM103362. Partial funding was obtained through a PhD grant to JLBN (SFRH/BD/51477/2011) from the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Program and from national funds through FCT - Foundation for Science and Technology - under the project PEst-C/MAR/LA0015/2013 and by the projects MARBIOTECH (reference NORTE-07-0124-FEDER-000047) within the SR&TD Integrated Program MARVALOR - Building research and innovation capacity for improved management and valorization of marine resources, supported by the Programa Operacional Regional do Norte (ON.2-O Novo Norte) and NOVOMAR. The sample collection in Cape Verde was supported by Fundação Calouste Gulbenkian. We thank Dr. Grzegorz Bulaj from the

University of Utah for creative discussions on the peptide synthesis, Dr. William Low from the Salk Institute for Biological Studies, La Jolla, CA and Dr. Krishna Parsawar from the University of Utah HSC Cores for mass spectrometry, Dr. Scott Endicott from the University of Utah HSC Cores for amino acid analysis, Dr. Manuel B. Aguilar from the Universidad Nacional Autonoma de Mexico for the test peptide sequencing run by Edman degradation, and My Huynh and Terry Merritt for help with the figures and manuscript.

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Chapter 4

Small Molecules in the Cone Snail Arsenal

4.Small Molecules in the Cone Snail Arsenal

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Small Molecules in the Cone Snail Arsenal

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Supporting Information

Abstract

Cone snails are renowned for producing peptide-based venom, containing conopeptides and conotoxins, to capture their prey. A novel small-molecule guanine derivative with unprecedented features, genuanine, was isolated from the venom of two cone snail species. Genuanine causes paralysis in mice, indicating that small molecules and not just polypeptides may contribute to the activity of cone snail venom.

Marine mollusks of the genus *Conus* produce bioactive peptides that are used in medicine.[1] Each snail synthesizes an array of different peptides, each of which targets a specific receptor or ion channel subtype, often with exquisite selectivity. The combination of these peptides, referred to as a “cabal”, creates a unique response in prey animals, incapacitating prey and enabling consumption by the cone snail.[2] Although cone snails eat fish, polychaete worms, or other mollusks, because of the striking conservation of many ion channels and receptors across higher animals, the peptides are often highly selective to human proteins as well.

Because of these properties, cone snail research has focused on venom peptides, with good reason. Recently, we found potent neuroactivity in a venom fraction containing small molecules. Here, we describe the discovery of a novel guanine derivative, genuanine, which induces paralysis in mice. Given the extensive previous chemical characterization in cones, even in one of the species described below, it is remarkable

that this bioactive guanine derivative with novel modifications of the purine ring was never characterized prior to the present study. Natural small molecule derivatives of nucleic acids, such as cytosine arabinoside, often have novel biological activity, sometimes with important biomedical applications.

Conus genuanus was collected in São Vicente, Cape Verde. *C. genuanus* belongs to a small number of cones with a pigmented venom duct, prompting us to search for small molecule pigments, which have not yet been characterized in cones. The red pigment is asymmetrically distributed when the venom is *in situ* in the venom duct of the snail. Dry venom collected from the red portion of the venom duct was extracted, providing a mixture of small molecules that was neuroactive when injected into mice. Careful examination of the venom components indicated several compounds, including unstable pigments that are under investigation. However, one of these, genuanine, was stable and exhibited a very similar paralytic activity in mice to what was seen in the crude small-molecule fraction.

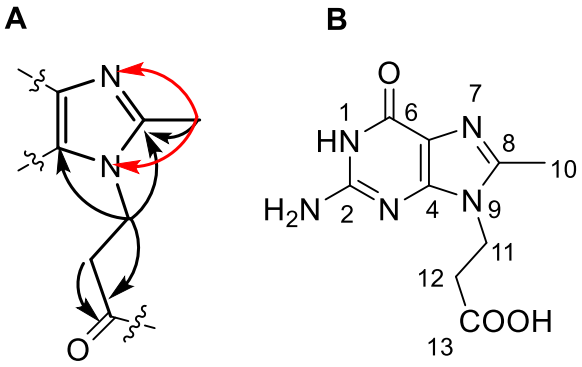

Figure 1: Structure of genuanine. A) Key HMBC data used to determine the right ring substructure. Red arrows: 15N-HMBC; black arrows: 13C-HMBC. B) Structure of genuanine.

Genuanine was isolated as a colorless solid (1 mg). Solubility issues and the small amount of available material limited the NMR analysis. The ^{1}H NMR spectrum showed only two methylene triplets (∂ 3.00, 4.43 ppm, t, J = 7.5 Hz) and one methyl singlet (∂ 2.74 ppm, s). An HSQC spectrum indicated chemical shifts consistent with adjacency of the methylenes to nitrogen and the methyl to an aromatic carbon. Indeed, a ^{15}N-HMBC spectrum revealed connectivity to two nitrogen units, as well as the proximity of the methylene and methyl groups. A ^{13}C-HMBC experiment indicated the substructure as shown in Figure 1a.

Despite insufficient NMR data, the molecular mass (m/z = 238.0935) and a characteristic UV signature led us to speculate that the genuanine was a derivative of guanine (Figure 1b). Genuanine was synthesized from guanine in two steps. The

synthetic material initially appeared on the basis of ^{1}H NMR spectroscopy to be different from the natural genuanine. However, upon mixing the natural and synthetic material together, it became apparent that the two are identical (Figure S1 3.11 and 3.12), but that chemical shifts are extremely sensitive to slight changes in conditions (Figure S2). Combination of synthetic and natural genuanine in 35% DCl enabled measurement of a ^{13}C spectrum, providing further evidence of the structure as shown. Thus, the structure of genuanine was simultaneously elucidated and confirmed.

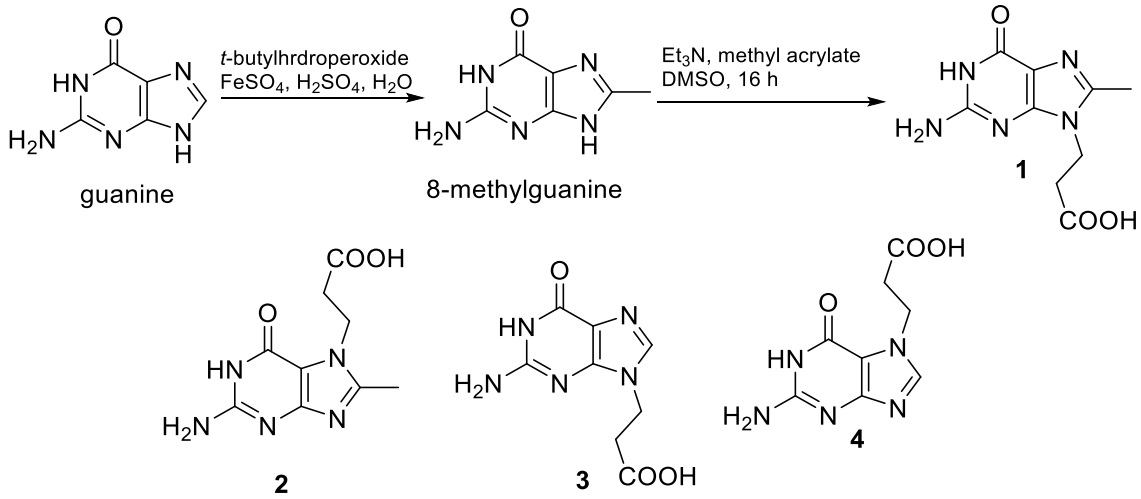

Figure 2: Synthesis of genuanine (top)[3] and synthetic isomers used in biological assays (bottom).

Compound **1** exhibited profound activity at doses as low as 40 nmol per mouse, in which the mouse was paralyzed in all 4 limbs for >2 h. However, best activity was obtained with fresh samples, and activity decreased with storage time (see Supporting Information for a complete table of all conditions and doses tested). This activity mimicked the potent paralytic activity found in the venom extract. The reason for this loss of activity over time has yet to be elucidated, but it was reproducible with both natural and synthetic **1**.

Initial attempts to improve synthetic yield also led to an array of regioisomers of genuanine, compounds **2**-**4** (Figure 2), for which no activity was detected (Table S1), suggesting a possible structure-activity relationship underlying neuroactivity.. Other nucleic acid analogs with neuroactivity have previously been described. For example, a series of 8-oxoisoguanines from sponges inhibit GABAergic transmission in mice, while a sea anemone adenosine derivative caissarone is an adenosine receptor antagonist.[4] In the synthetic arena, guanine derivatives have been synthesized as purinergic receptor agonists and antagonists that act centrally,[5] among many other activities. It is

highly speculative to connect any of these previous results to our observed activity here, but they provide further confidence that nucleic acids can affect paralysis and the CNS. It is noteworthy that genuanine is present in sufficient quantity to exert the same effect in the crude venom. Thus, we propose that **1** is an active constituent in cone snail prey-capturing arsenal, and not just a passive ingredient in the venom duct.

Venom peptides vary greatly between cone snail species,[1b] so we sought to determine whether genuanine was widespread or restricted to *C. genuanus*. At random, we selected 9 additional cone snails collected from various locations in the Pacific Ocean for chemical analysis. We also selected *C. imperialis* from Oahu, Hawaii. While *C. imperialis* is not closely related to *C. genuanus*, it shares in common a pigmented venom duct (Figure 3) and to the best of our knowledge is the only other cone with a similar venom duct, having 2 regions of different colors. Only *C. genuanus* and *C. imperialis* contained genuanine. Other venom duct extracts were rich in nucleic acids, but these were the primary metabolites guanine and hypoxanthine.

Figure 3: Out of the ~800 known species of cone snails, *C. imperialis* (left) and *C. genuanus* (center) have the rare property of venom ducts containing red pigment at the distal end (right).

Recently, it has been shown that *Conus geographus* venom peptides vary in different regions of the venom duct.[6] Similarly, in *C. genuanus*, the red (distal) and yellow (proximal) venom duct products are very different (Figures 4 and S6). While the proximal venom duct contains largely peptidic, high-molecular weight components, the distal venom duct is dominated by low-molecular weight materials, of which genuanine **1** is the major stable metabolite.

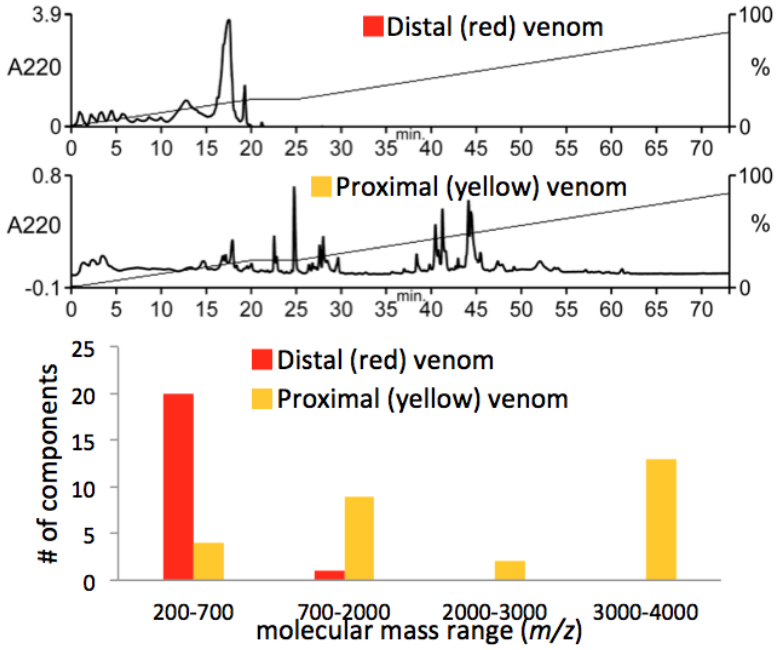

Figure 4: **Venom components from *C. genuanus*. HPLC-UV traces show that the distal and proximal venom ducts contain quite different components (top). Enumeration of major peaks in HPLC-MS spectra show that, in contrast to the proximal duct the distal duct is dominated by small molecules (bottom).**

Genuanine belongs to a rare family of C-8 methylated nucleic acid derivatives. Nucleosides are methylated at C-8 by radical alkylators, such as procarbazine.[7] In nature, a C-8 methylated adenosine derivative is known as a component of bacteria ribosomal RNA;[8] its biosynthesis utilizes radical SAM enzymes.[9] To the best of our knowledge, these compounds were not known from eukaryotes, nor was the methylated guanine or guanosine known in nature. Genuanine is also modified by propionate on N-9, which is a previously unknown modification of nucleic acids. Speculatively, this group could possibly also be added from methionine via a radical SAM enzyme, among other possible routes, possibly based upon tRNA metabolism. Alternatively, in other mollusks pigmented organs are associated with oxidative / radical metabolism[10] consistent with C-8 modification. A large family of nucleic acid analogs from marine animals. Most recently, several nucleosides from a sponge were produced by bacteria cultivated from the same sponge.[11] Both cone snails and their associated bacteria are known to produce compounds,[12] but determination of the ultimate source of genuanine awaits experiment.

The finding of paralytic small molecules as venom components in cone snails was unanticipated. To the best of our knowledge, only one previous report documents a small molecule: serotonin was found in small amounts in the venom duct of *C.*

imperialis.[13] Thus, it appears that small molecules are important and previously unrecognized contributors to the toxicity of cone snail venom.

Associated content

Supporting Information

Supporting Information. Materials and methods, NMR and MS spectra. The Supporting Information is available free of charge on the ACS Publications website at

DOI: 10.1021/acs.orglett.5b02389

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Acknowledgment

This project was funded by NIH R01GM107557 to EWS, NIGMS P01GM048677 to BMO, NIH ICBG U01TW008163 to EWS and BMO, and UID/Multi/04423/2013, MARBIOTECH and NOVOMAR. We thank J. Cox of the Utah Mass Spectrometry and Proteomics Core for mass measurements reported in this study; and the Foundation for Science and Technology (FCT - SFRH/BD/51477/2011), Fundação Calouste Gulbenkian and University of Cape Verde (UniCV) for sample collection support.

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Supporting Information for

Small molecules in the cone snail arsenal

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1. Experimental section

1.1. General experimental procedures

NMR data were collected using a Varian INOVA 600 (^{1}H 600 MHz, ^{13}C 150 MHz) NMR spectrometer equipped with a 5 mm ^{1}H[^{13}C,^{15}N] triple resonance cold probe with a z-axis gradient, utilizing residual solvent signals for referencing. High-resolution ESIMS analyses were performed on a Bruker (Billerica, MA) APEXII FTICR mass spectrometer equipped with an actively shielded 9.4 T superconducting magnet (Magnex Scientific Ltd., UK) and an external Bruker APOLLO nanospray ESI source. Typically, a 5 µL sample was loaded into the nanoelectrospray tip (New Objective, Woburn, MA) with a potential of 1000 V applied between the nanoelectrospray tip and the capillary. Supelco (St. Louis, MO) Discover HS (4.6 × 150 mm) and semipreparative (10 × 150 mm) C_{18} (5 µm) columns were used for analytical and semipreparative HPLC, respectively, as conducted on a Hitachi (Dallas, TX) Elite Lachrom System equipped with a Diode Array L-2455 detector.

1.2. Extraction and isolation

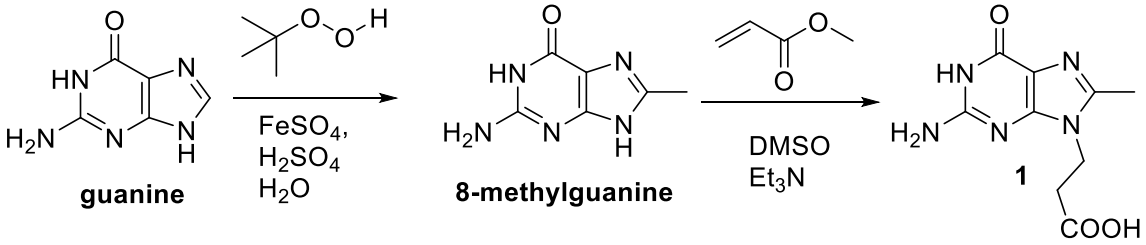
Conus genuanus specimens (55–65 mm shell length) were collected by SCUBA diving at 10-15 m depths in west coast of São Vicente Island, Cape Verde Archipelago. The specimens are predominantly buried in the sand and under rocks. Animals were dissected, and a red portion of the venom duct was cut off. The venom was squeezed out by forceps, lyophilized and stored at -80°C. Dry venom powder (20 mg) was extracted three times with H_2O (1 mL). The H_2O extract was purified on C18 HPLC with mobile phase 1% MeCN in H_2O (0.05% TFA was added to MeCN) to obtain compound 1 (1.0 mg).

Genuanine (1): white solid; UV (MeOH) λ_{max} 203, 254, 280 nm; ^{1}H and ^{13}C NMR (see Table 1); HRESIMS *m/z* 238.0936 $[M+H]^{+}$ (calcd for $C_9H_{11}N_5O_3$, 238.0935).

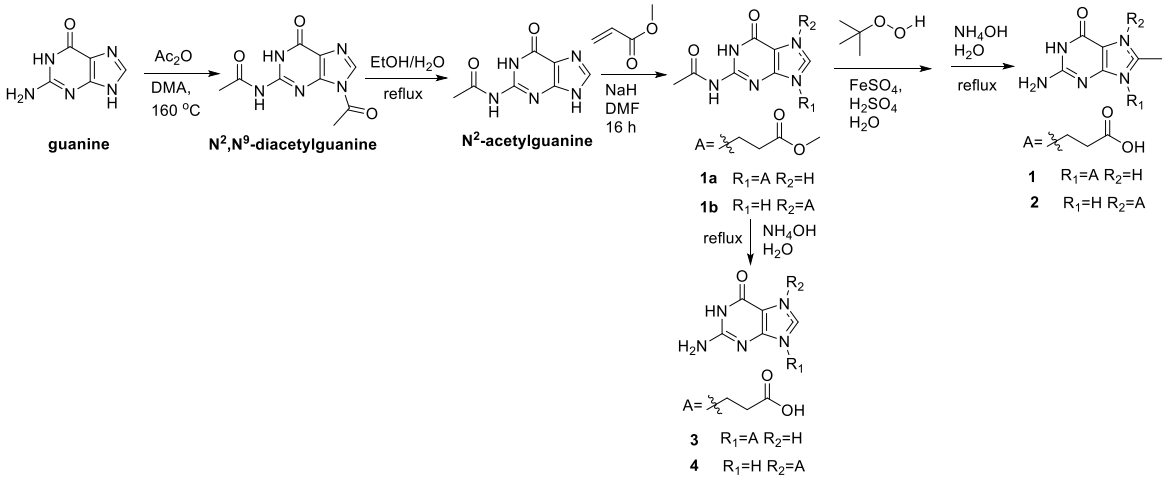
1.3. Preparation of substituted guanines

8-Methylguanine was prepared by free radical methylation of guanine in acidic aqueous solution as described by Maeda et al.[1] Guanine (150 mg, 1.0 mmol) and $FeSO_4 \times 7H_2O$ (1.2 g, 4.32 mmol) were added to 0.5 M H_2SO_4 (60 mL degassed with N_2), and the

mixture was stirred for 15 min under N_2. Subsequently, a 2% aqueous solution of *t*-butylhydroperoxide (20 mL) was added dropwise over 10 min at RT by syringe. After 1 h, the reaction mixture was neutralized with $NaCO_3$.



The resulting dark red sludge was mixed with silica gel and dried under vacuum. The silica gel was loaded on an open column and eluted with MeOH:$CHCl_3$ (1:1). The flow through was dried under vacuum to obtain crude 8-methylguanine, which was dissolved in 50 mL DMSO. The solution was heated to 60°C, followed by addition of triethylamine (120 µL) and methyl acrylate (90 µL). After stirring for 16 h at 60°C, EtOAc (100 mL) was added, and the resulting precipitate was filtered and washed with EtOAc. The solid was dissolved in H_2O and further purified by C18 HPLC to yield compound **1** (23 mg, 10% yield).



N^2-Acetylguanine was prepared following the method described by Vo et al.[2] N^2-Acetylguanine (230 mg) was dissolved in dimethylformamide (50 mL). NaH (48 mg) was added to the solution. The mixture was heated to 60°C and stirred for 1 h, followed by addition of methyl acrylate (106 µL). After stirring for 16 h at 60°C, EtOAc (100 mL) was added into the reaction mixture, and the resulting precipitate was filtered and washed with EtOAc to yield 198 mg (yield 60%) of a mixture of compounds **1a** (70%) and **1b** (30%) (by LC-MS). The mixture (10.0 mg) of **1a** and **1b** was methylated by the

free radical method described above. The reaction mixture was neutralized with Na_2CO_3. The resulting dark red sludge was mixed with silica gel and dried under vacuum. The silica gel was loaded on an open column and eluted with MeOH:$CHCl_3$ (1:1). The flow through was dried under vacuum to obtain a mixture of methylated **1a** and **1b**, which was subsequently hydrolyzed by adding 27% NH_4OH (1 mL) and MeOH (10 mL) followed by reflux overnight to yield compounds **1** (3.8 mg, 63%) and **2** (1.5 mg, 58%). The mixture of **1a** and **1b** (10.0 mg) was directly hydrolyzed in 27% NH_4OH (1 mL) and MeOH (10 mL) to yield compounds **3**[3] (5.0 mg, 89%) and **4**[4] (2.1 mg 87%).

Compound 2, white solid; UV (MeOH) λ_{max} 203, 254, 278 nm; HRESI-MS: *m / z* 238.0939 $[M+H]^+$ (calcd for $C_9H_{11}N_5O_3$, 238.0935); ^{1}H NMR (DCl 35% v/v in D_2O, 500 MHz, ref H_2O 4.9 ppm): δ 0.42 (t, 2H, H-11), -1.08 (t, 2H, H-12), -1.30 (t, 3H, H-10). ^{13}C NMR (DCl 35% v/v in D_2O, 125 MHz): δ 9.0, 30.4, 40.0, 105.4, 133.3, 146.7, 147.5, 147.7, 170.5.

Compound 3, white solid; UV (MeOH) λ_{max} 203, 254, 278 nm; ESI-MS: *m / z* 224 $[M+H]^+$; ^{1}H NMR (D_2O , 500 MHz): δ 8.80 (s, 1H, H-8), 4.37 (t, 2H, H-11), 2.89 (t, 2H, H-12). ^{13}C NMR (D_2O, 125 MHz): δ 9.0, 32.5, 40.8, 107.6, 137.7, 150.2, 174.3.

Compound 4, white solid. UV (MeOH) λ_{max} 203, 254, 280 nm; ESI-MS: *m / z* 224 $[M+H]^+$; ^{1}H NMR (D_2O , 500 MHz): δ 8.54 (s, 1H, H-8), 4.50 (t, 2H, H-11), 2.86 (t, 2H, H-12). ^{13}C NMR (D_2O, 125 MHz): δ 36.4, 46.5, 110.1, 124.8, 152.4, 176.0.

1.4. Biological activity

An aliquot of crude venom extract or pure synthetic or natural compound was resuspended in 12 µL of normal saline solution (NSS, 0.9% NaCl). Fifteen-day-old Swiss Webster mice (6-8 g of body weight) were intracranially injected using a 0.3 mL insulin syringe, and the same volume of NSS was injected in control mice. Mouse response was followed visually. The use of Swiss Webster mice followed protocols approved by the University of Utah Institutional Animal Care and Use Committee that conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.[5]

2. Table S1. The mice test results for compounds 1-4. (See Figure S6 for data with crude venom fractions). All observed activities were reversible, and mice recovered from the effect

Source	Assay date	Time from sample preparation	Dose	Number of mice	Observation
Natural **1**	April 15, 2015	same day	40 nmol	1	4 limbs paralyzed, mouse resting on belly > 2 h
Natural **1**	July 30, 2015	3 months	60 nmol	1	Mild sedation
Synthetic **1**	July 30, 2015	same day	30, 60, 180 nmol	3, 3, 2	No effects at 30 and 60 nmol; at 180 nmol, all limbs completely immobile and mice resting flat on belly for 45 min (1 mouse; and >2 h for the second mouse
Synthetic **2**-**4**	July 30, 2015	same day	30, 60, 180 nmol	18	No symptoms
Synthetic **1**	August 5, 2015	6 days	150, 300, 750 nmol	1, 1, 1	No symptoms for 150 and 300 nmol, at 750 nmol, all limbs completely immobile and mice resting flat on belly for 2 h
Synthetic **2**	August 5, 2015	6 days	180, 300	1, 1	No symptoms
Synthetic **1**	August 6, 2015	7 days	500, and 750 nmol	1, 1	500 nmol, difficulty in moving for >40 min 750 nmol: total paralysis >2 h.
Synthetic **1**	August 6, 2015	Same day	300, 500 nmol	1, 1	300 nmol: no activity 500 nmol: difficulty in moving for >40 min

3. Table S2. NMR data of natural 1 in D_2O

	^{1}H NMR[a] (ppm J in Hz)	^{13}C NMR[a] (ppm)	^{15}N NMR[b] (ppm)	^{1}H NMR[c] (ppm J in Hz)	^{13}C NMR[c] (ppm)	^{1}H NMR[d] (ppm J in Hz)	^{13}C NMR[d] (ppm)
4	-	152.6 C	-	-	ND		152.2 C
5							
6							153.3 C
7	-	-	88.3	-	-		
8	-	148.7 C	-	-	ND		152.5 C
9	-	-	32.6	-	-		
10	2.54 s	14.4 CH_3	-	2.54 s	14.4 CH_3	2.54 s	14.4 CH_3
11	4.29 t (7.5)	41.7 CH_2	-	4.23 t (7.5)	42.8 CH_2	4.21 t	44.3 CH_2
12	2.70 t (7.5)	38.4 CH_2	-	2.80 t (7.5)	35.5 CH_2	2.75 t	34.4 CH_2
13	-	178.9 C	-	-	ND		175.3 C

[a] Measured using the crude H_2O extract of the venom. [b]The chemical shifts of ^{15}N were obtained from ^{1}H-^{15}N HMBC in DMSO-d_6. [c]Measured using the HPLC-purified natural compound **1** with 0.05% TFA in mobile phase. [d]Measured in DCl 35% v/v in D_2O. The chemical shifts of a were referenced to the HDO peak; all other chemical shifts in b, c and d were referenced to the C-10 methyl peak of compound **1** in a.

4. Figure S1. NMR spectra of compounds 1-4

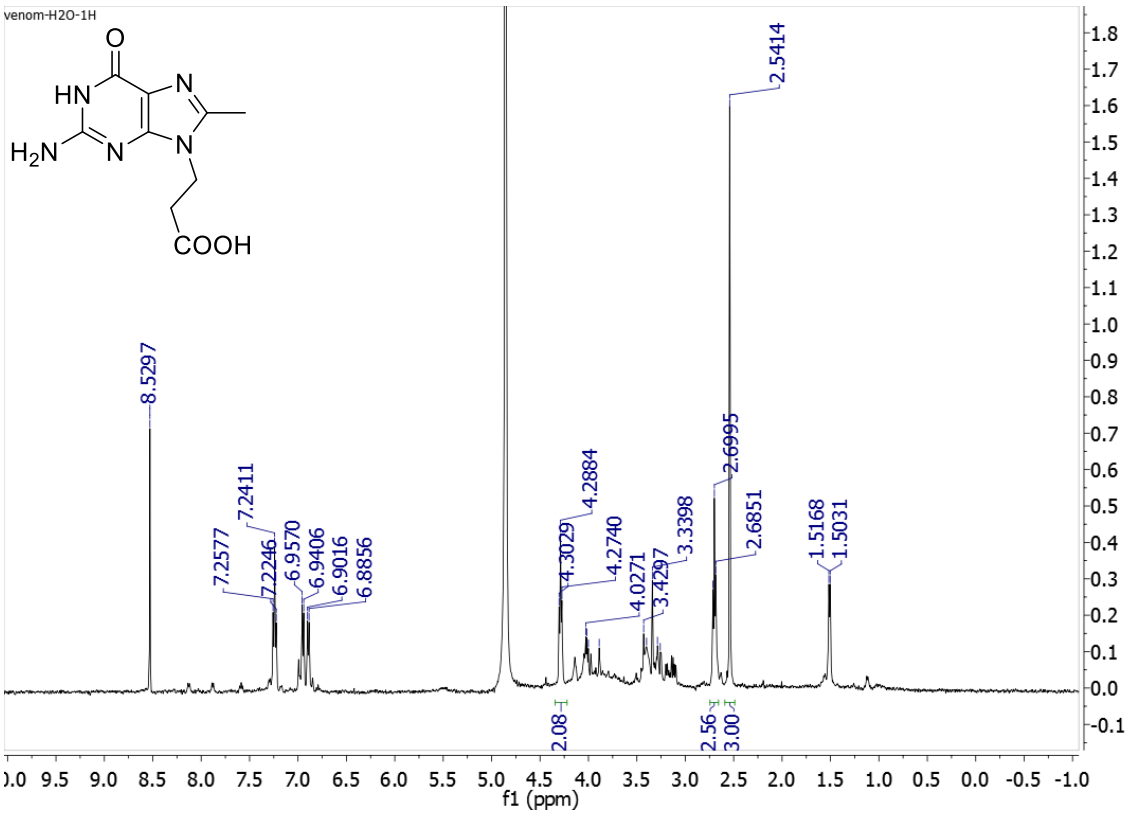


Fig. S1A ^{1}H NMR spectrum of compound 1 in D_2O in the crude H_2O extract of the venom.

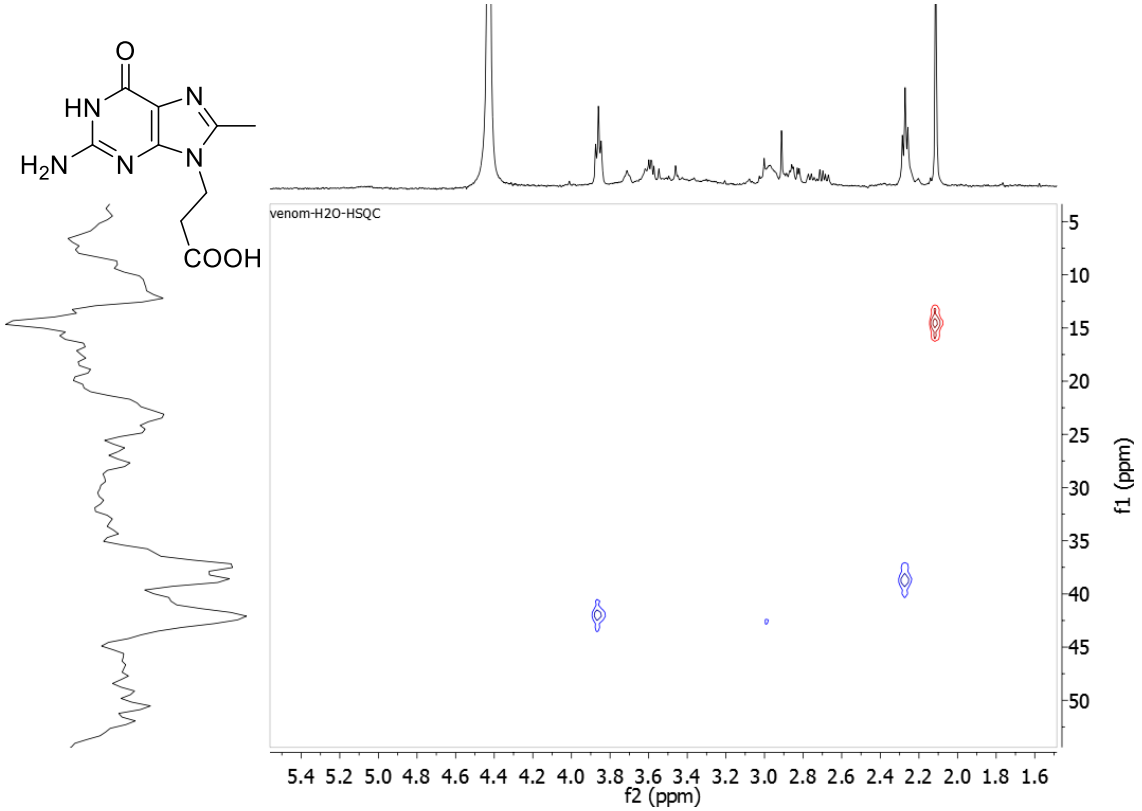


Fig. S1B HSQC spectrum of compound 1 in D_2O in the crude H_2O extract of the venom.

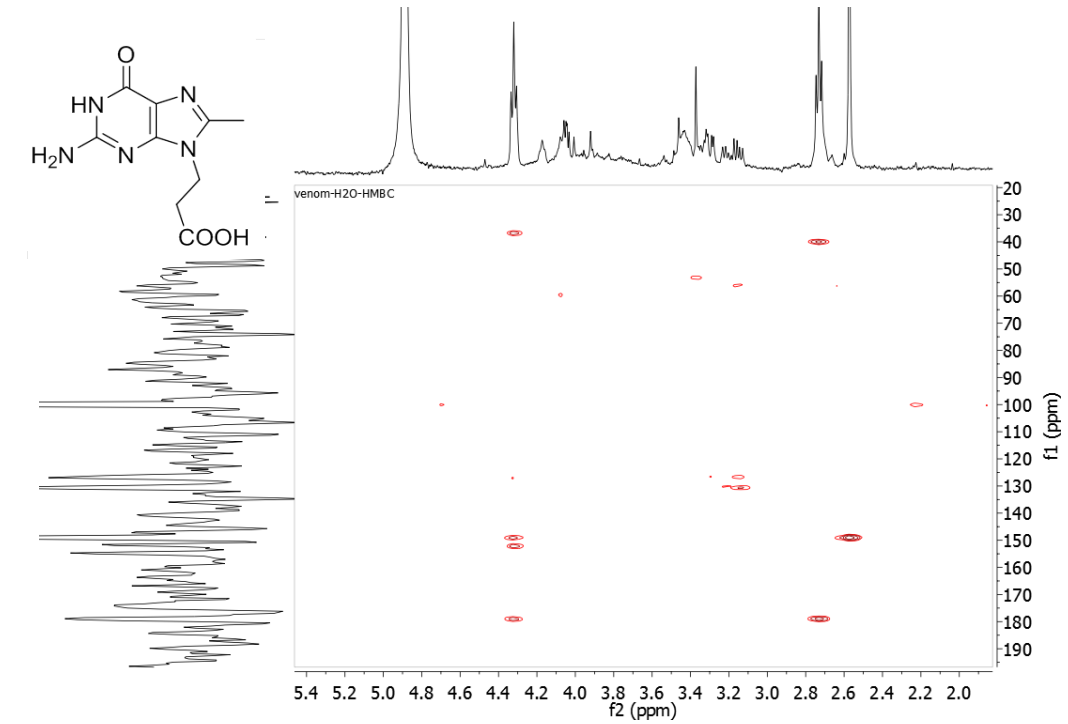


Fig. S1C HMBC spectrum of compound 1 in D_2O in the crude H_2O extract of the venom.

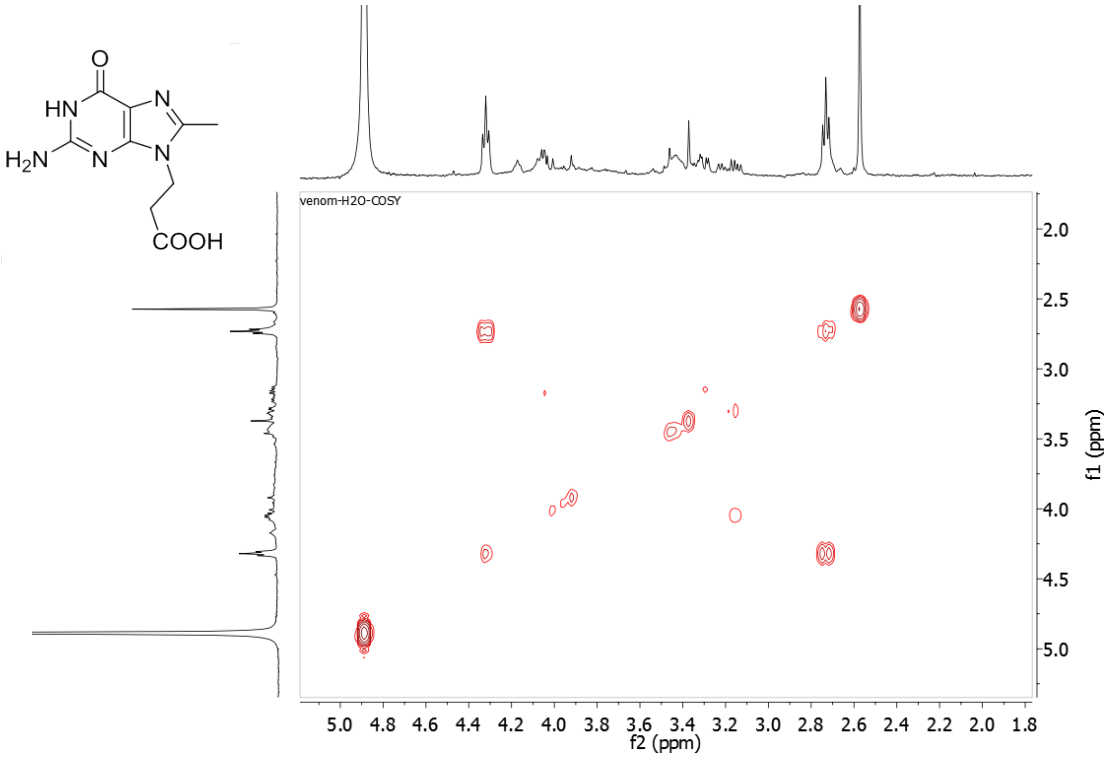


Fig. S1D ^{1}H-^{1}H COSY spectrum of compound 1 in D_2O in the crude H_2O extract of the venom.

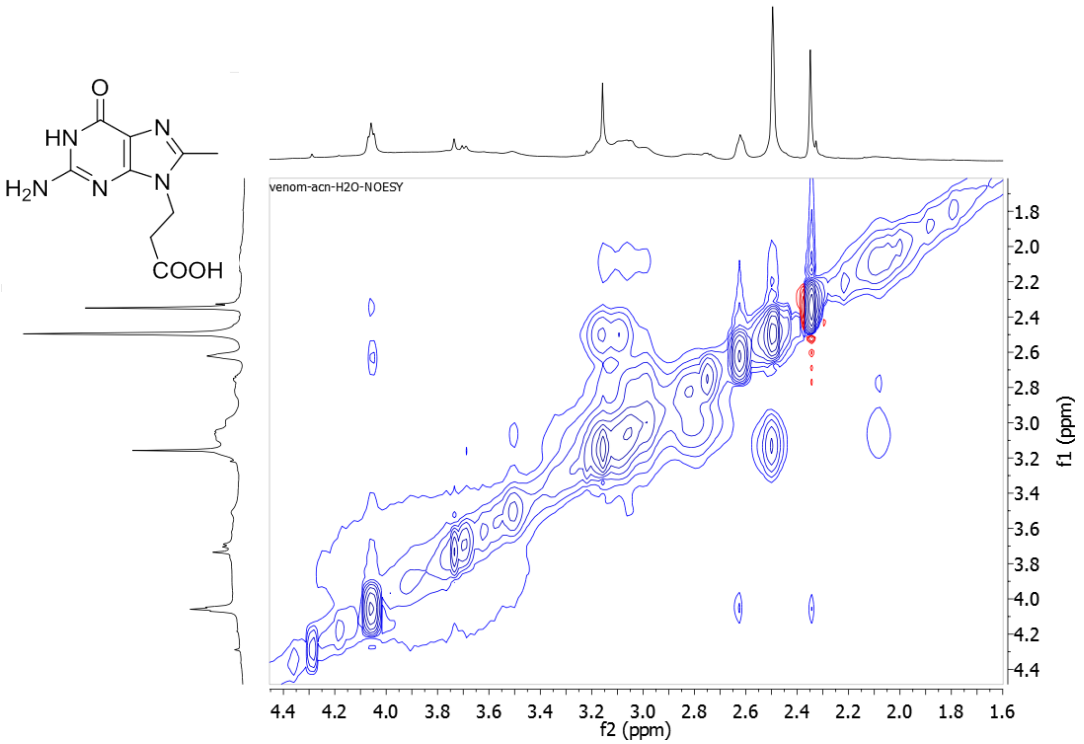


Fig. S1E NOESY spectrum of compound 1 in DMSO-d_6 in the crude H_2O extract of the venom.

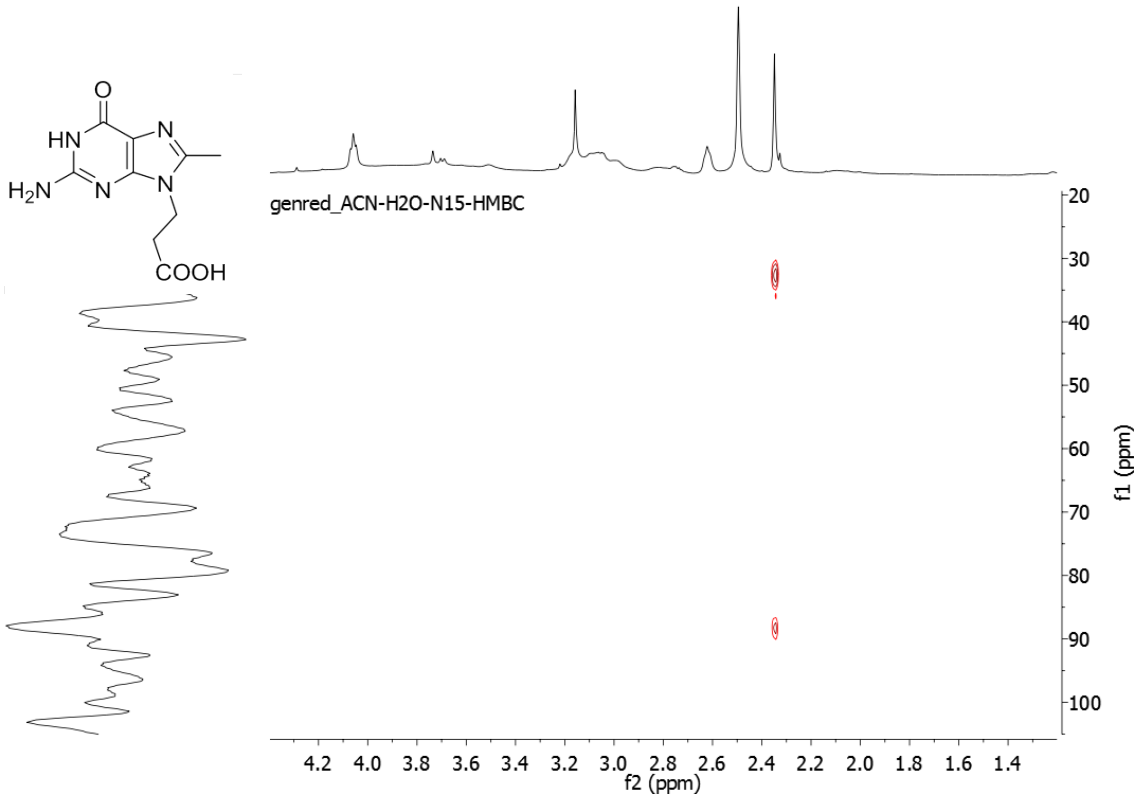


Fig. S1F ^{1}H-^{15}N HMBC spectrum of compound 1 in DMSO-d6 in the crude venom.

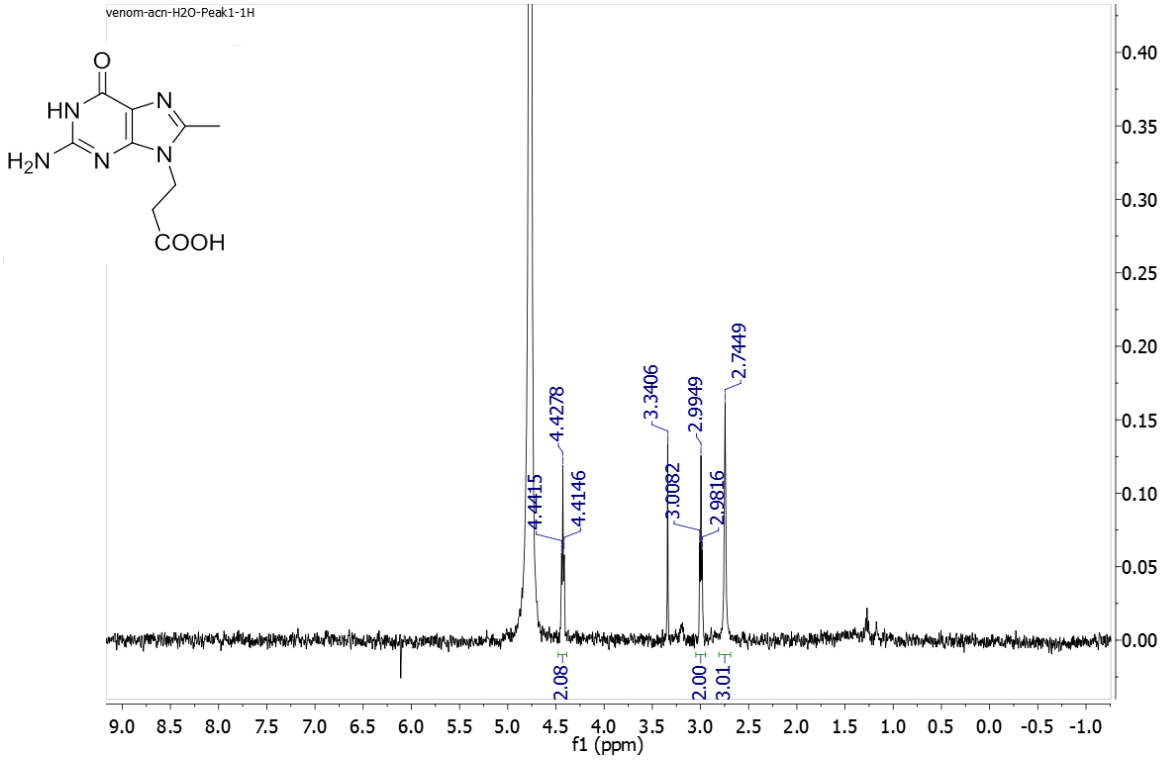


Fig. S1G ^{1}H NMR spectrum of purified compound 1 in D_2O.

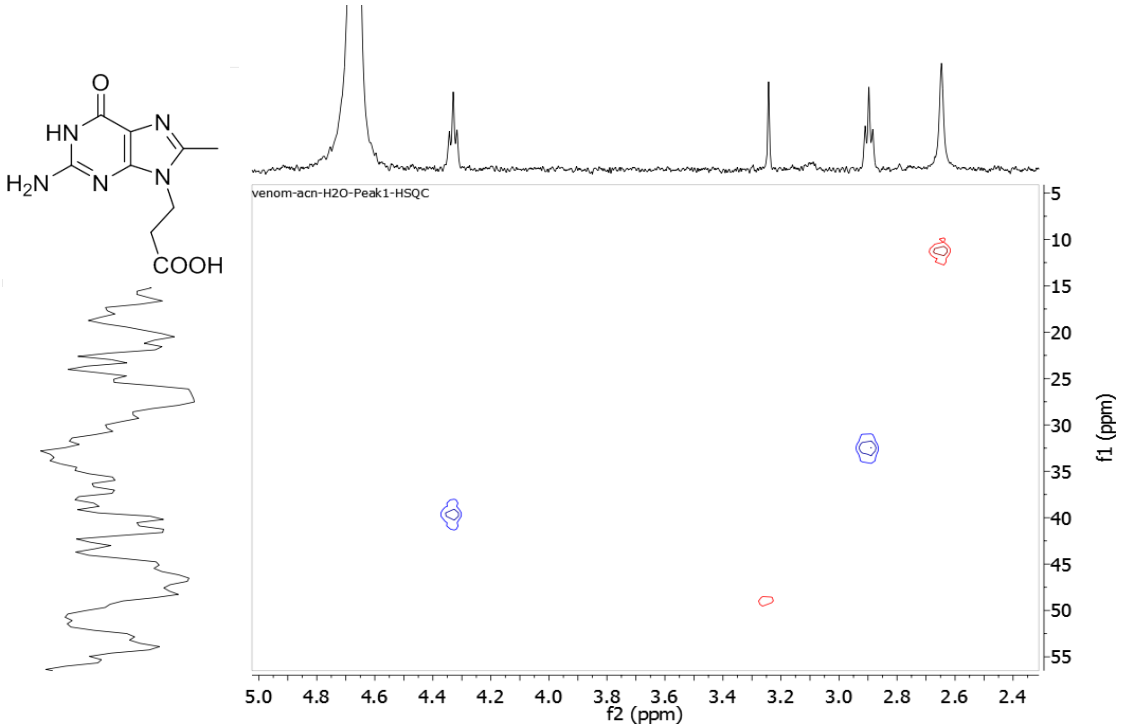


Fig. S1H HSQC spectrum of purified compound 1 in D_2O.

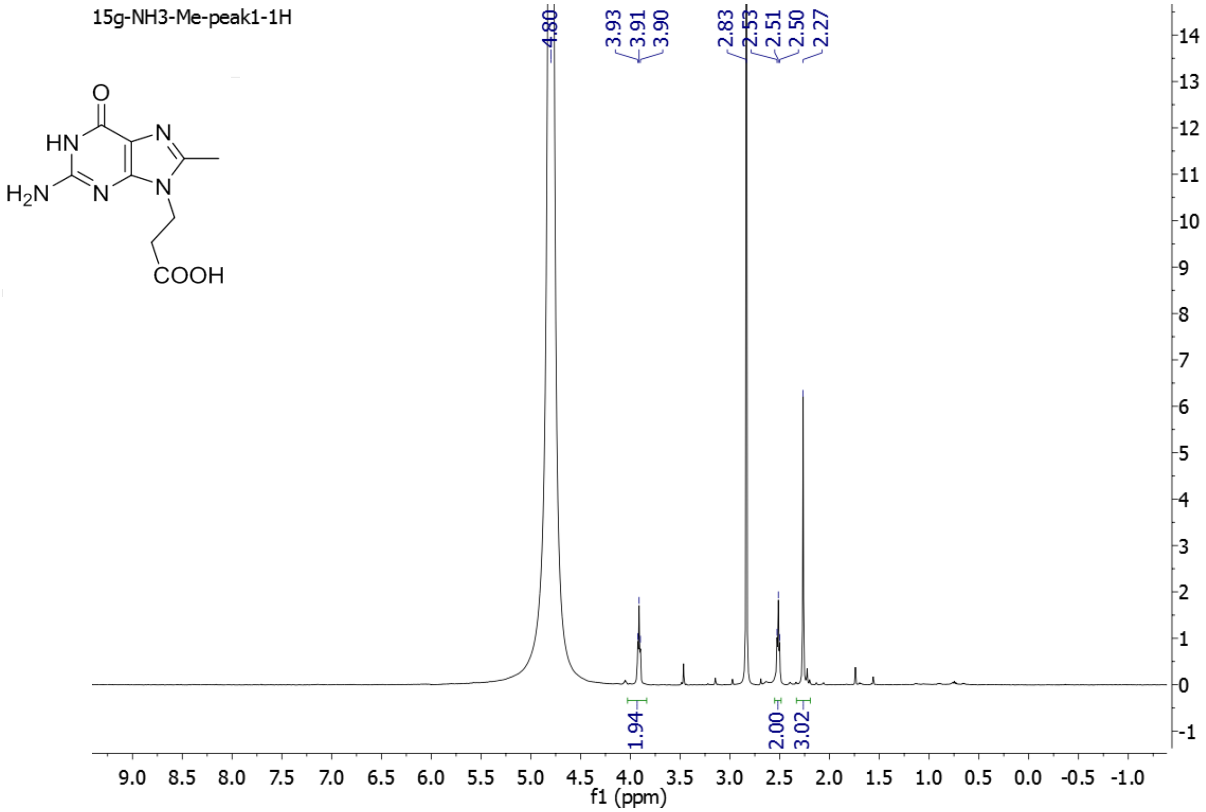


Fig. S1I ^{1}H NMR spectrum of synthetic compound 1 in D_2O.

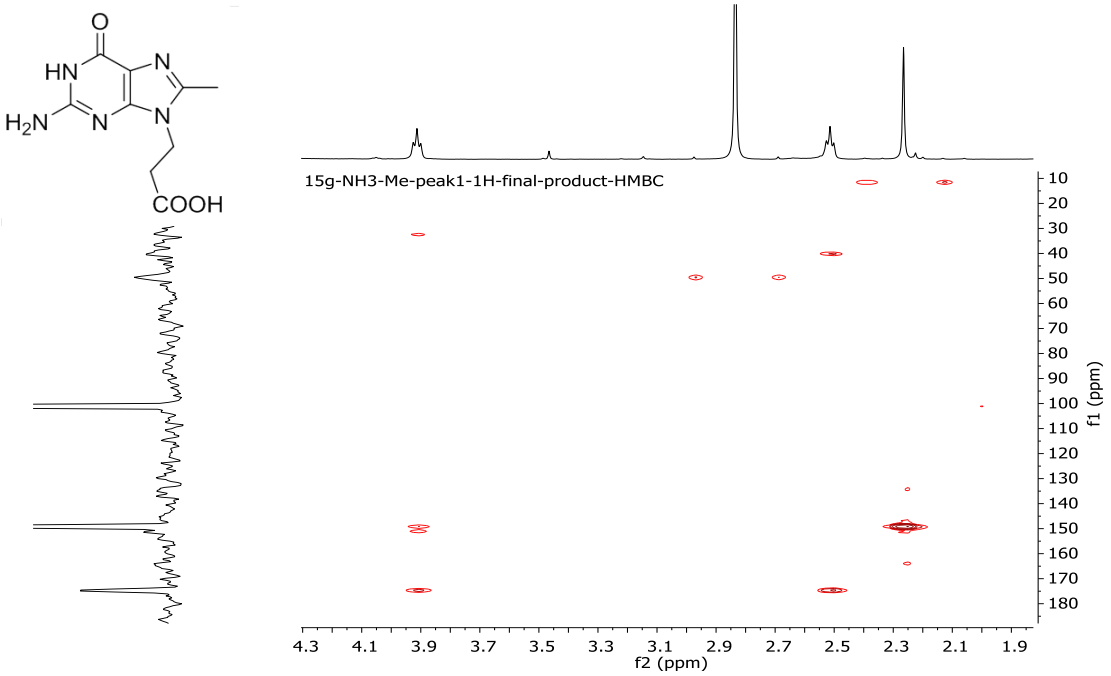


Fig. S1J HMBC spectrum of synthetic compound 1 in D_2O.

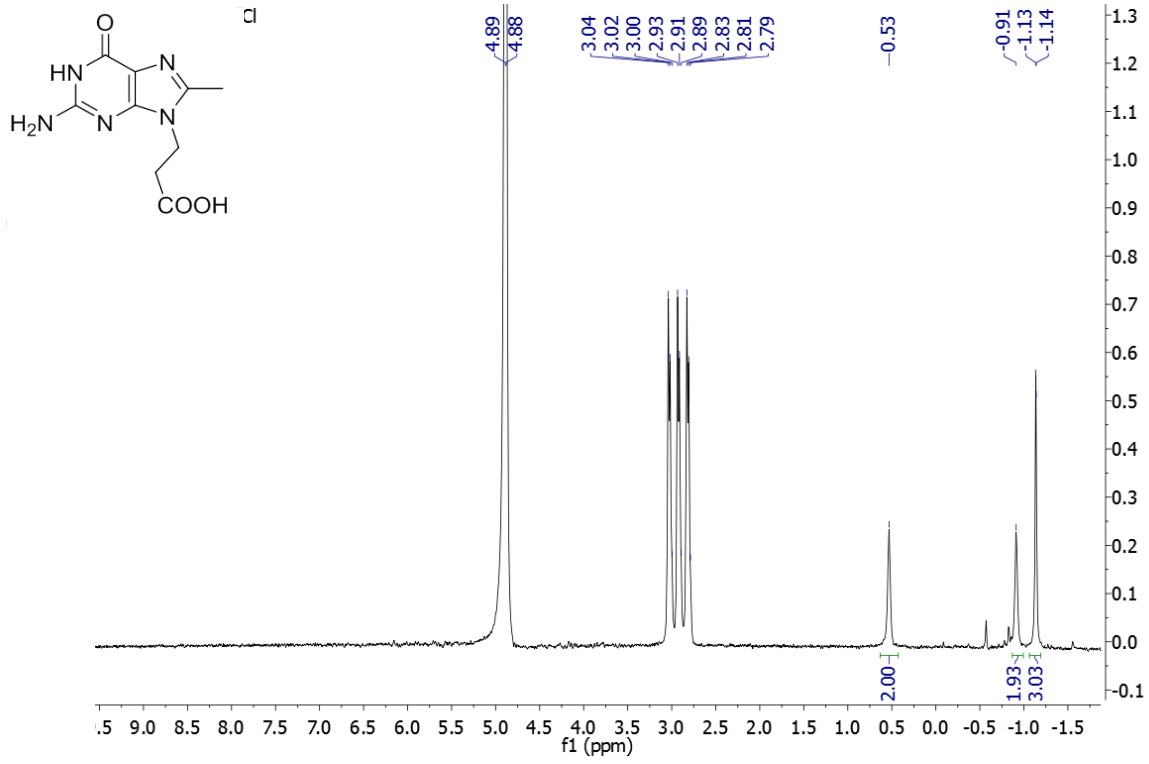


Fig. S1K ^{1}H NMR spectrum of mixture of natural genuanine (0.4 mg) and synthetic genuanine (0.4 mg) in DCl 35% v/v in D_2O.

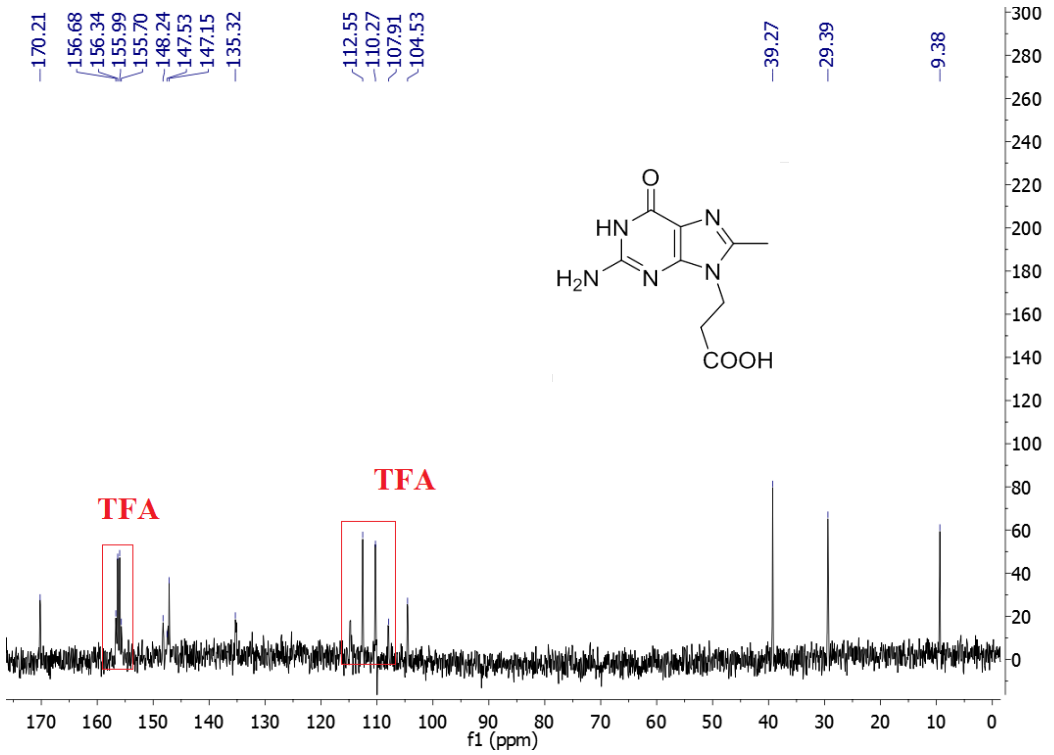


Fig. S1L ^{13}C NMR spectrum of mixture of natural genuanine (0.4 mg) and synthetic genuanine (0.4 mg) in DCl 35% v/v in D_2O.

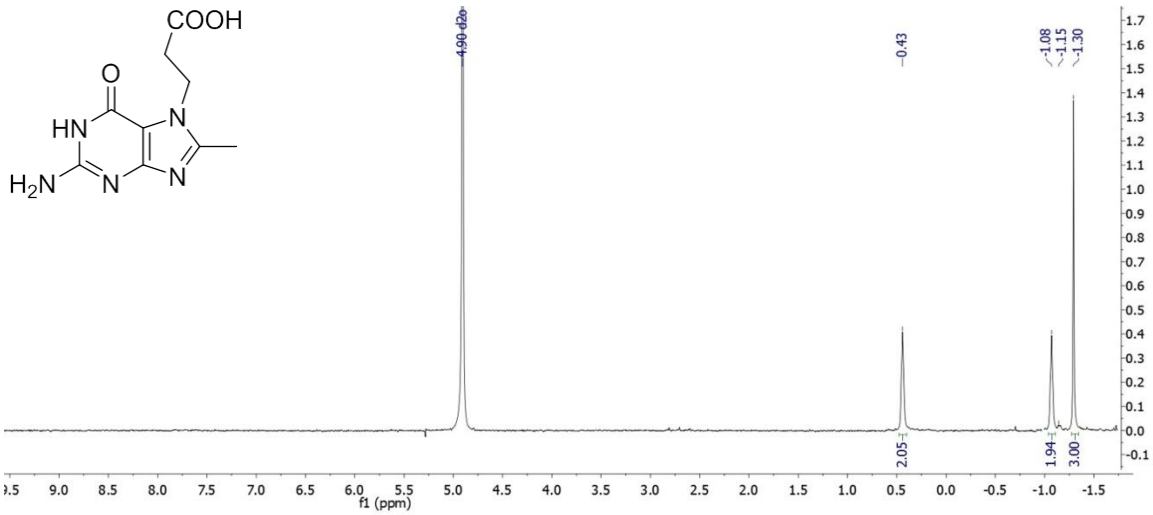


Fig. S1M ^{1}H NMR spectrum of 2 in DCl 35% v/v in D_2O.

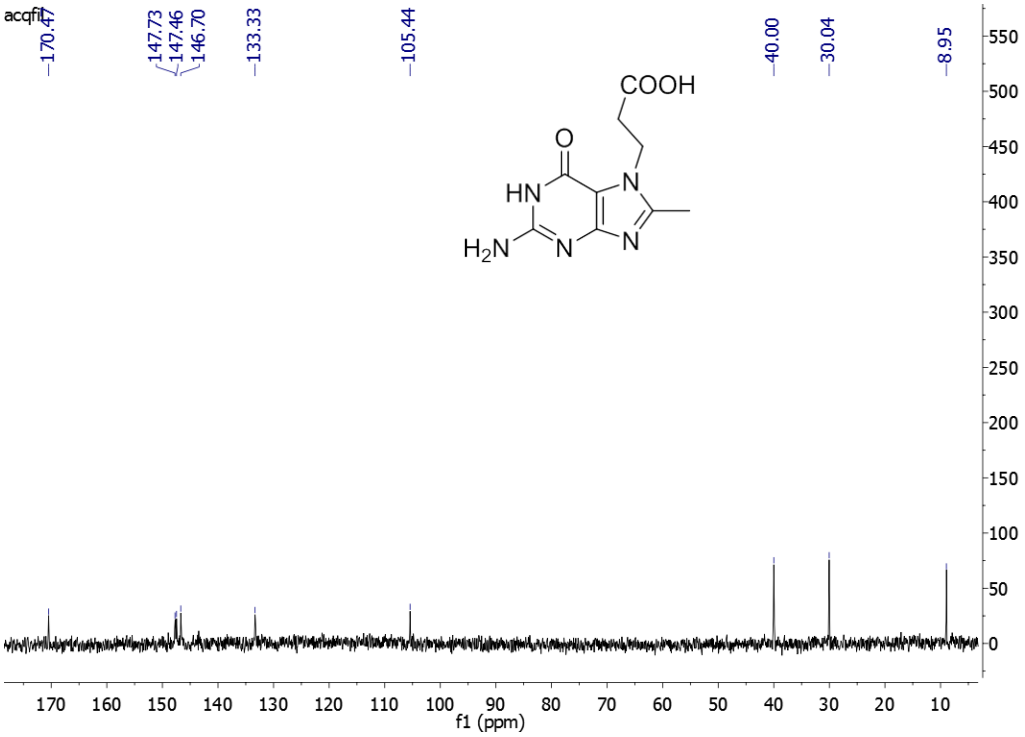


Fig. S1N ^{13}C NMR spectrum of 2 in DCl 35% v/v in D_2O.

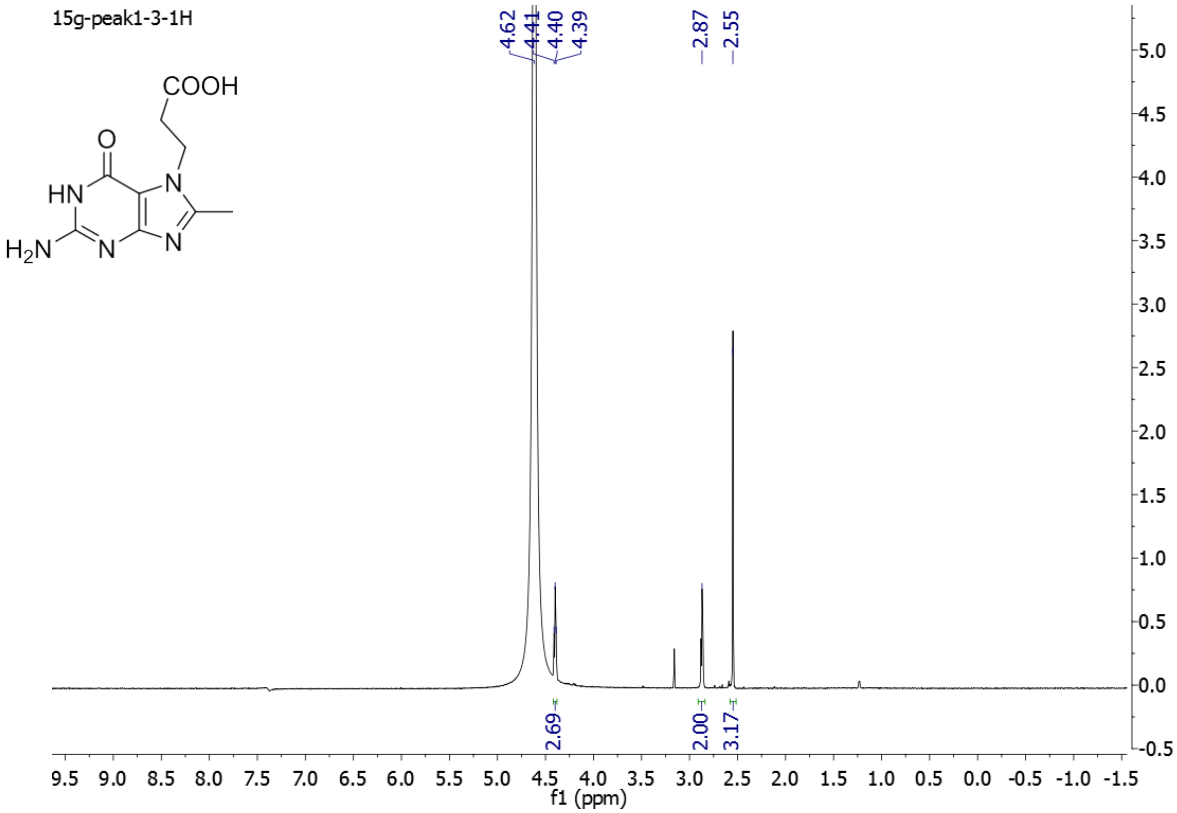


Fig. S1O 1H NMR spectrum of compound 2 in D2O.

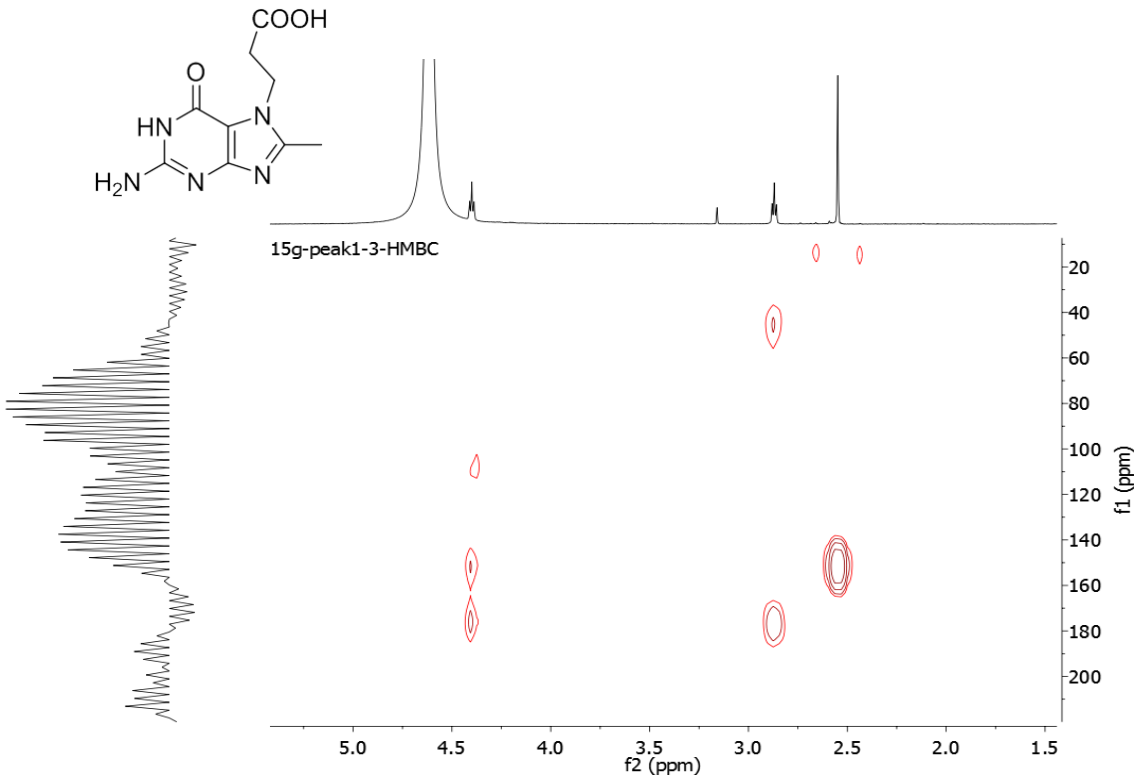


Fig. S1P HMBC spectrum of compound 2 in D2O.

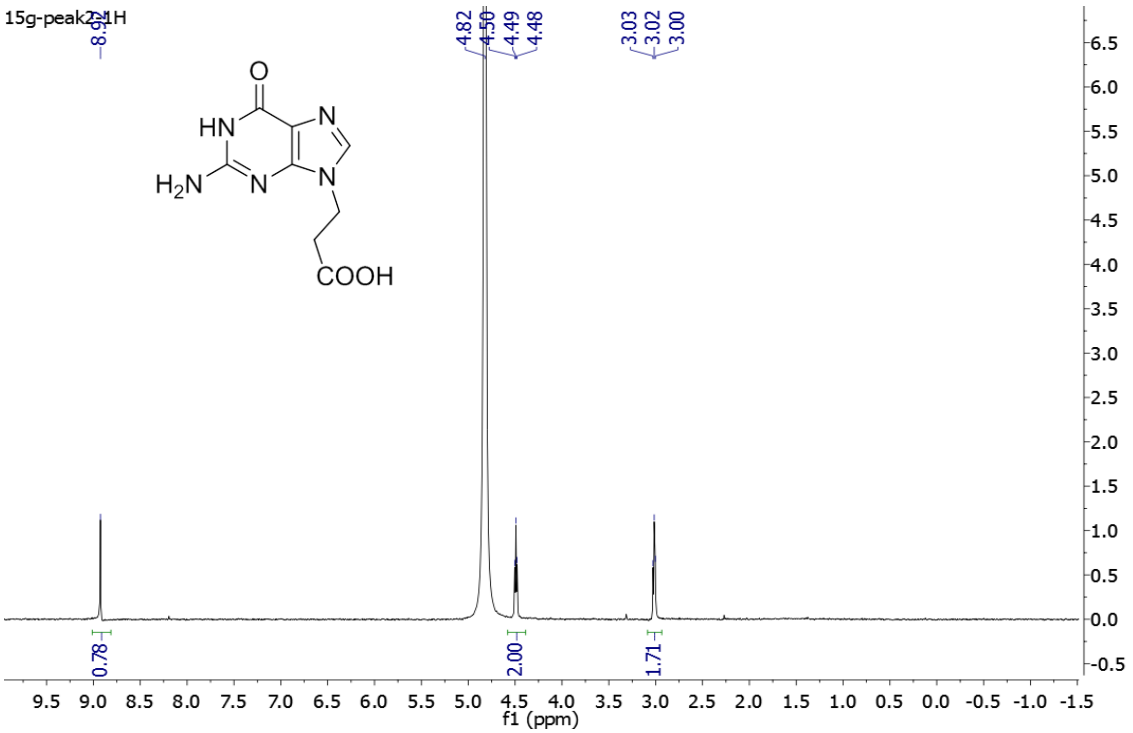


Fig. S1Q ^{1}H NMR spectrum of compound 3 in D_2O.

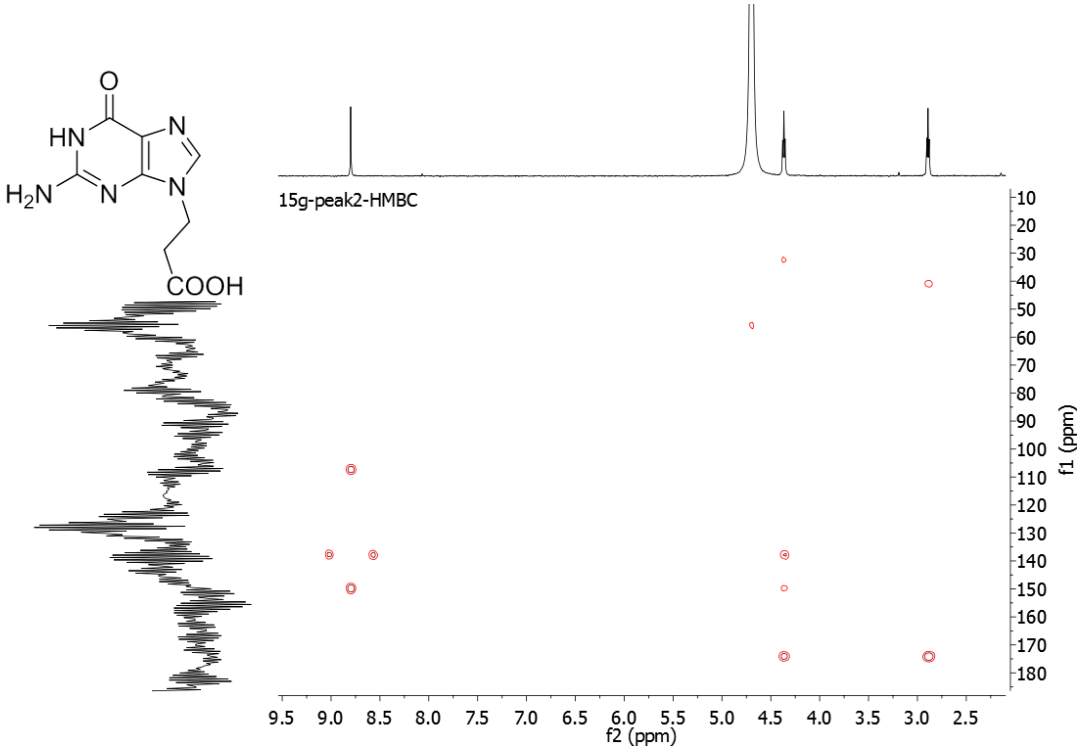


Fig. S1R HMBC spectrum of compound 3 in D_2O.

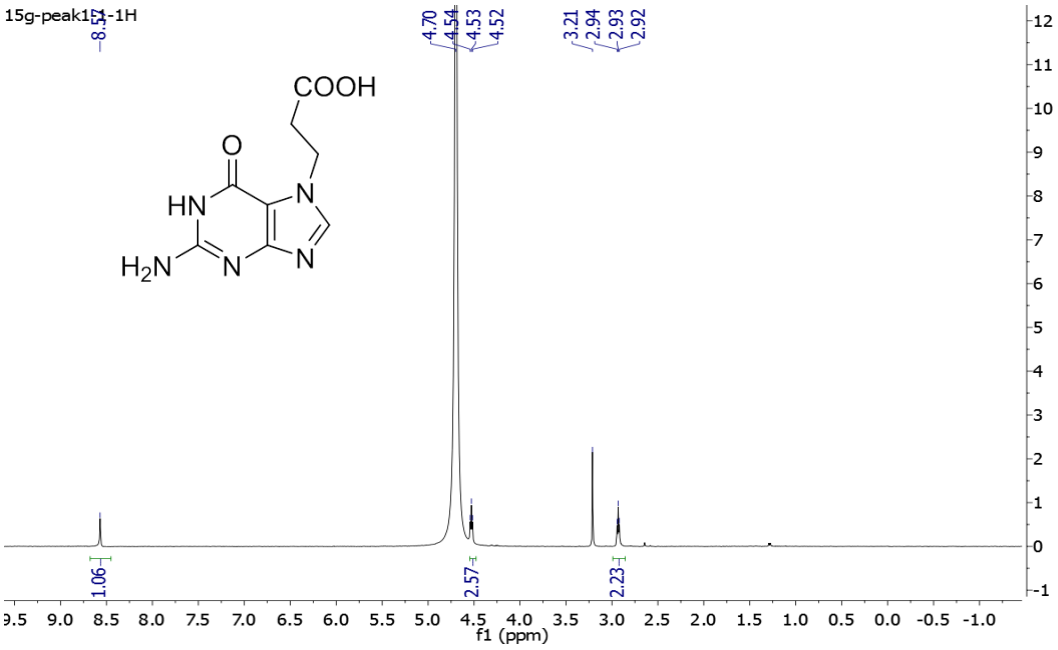


Fig. S1S ^{1}H NMR spectrum of compound 4 in D_2O.

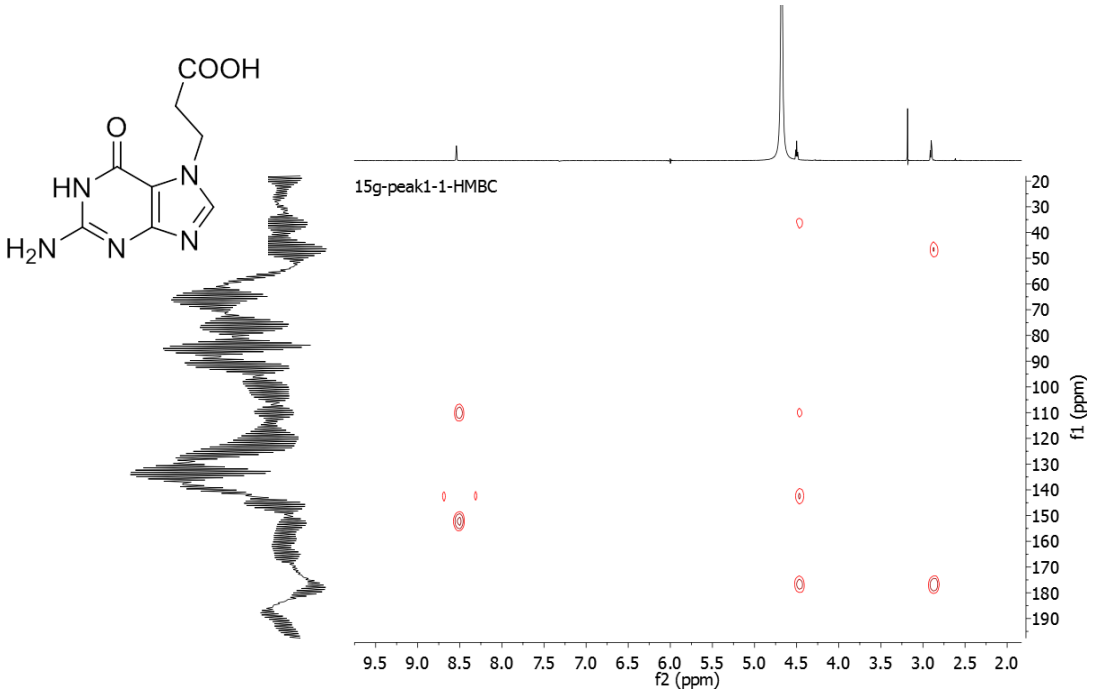
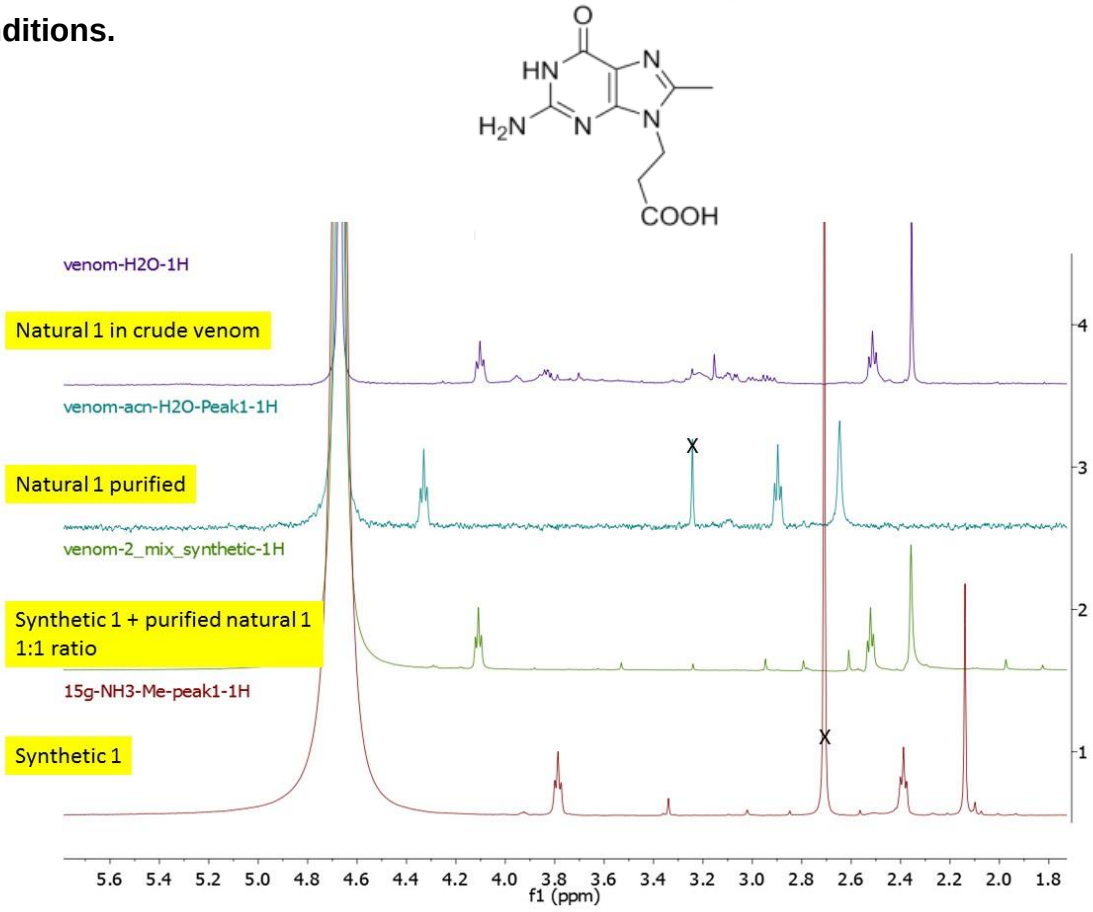
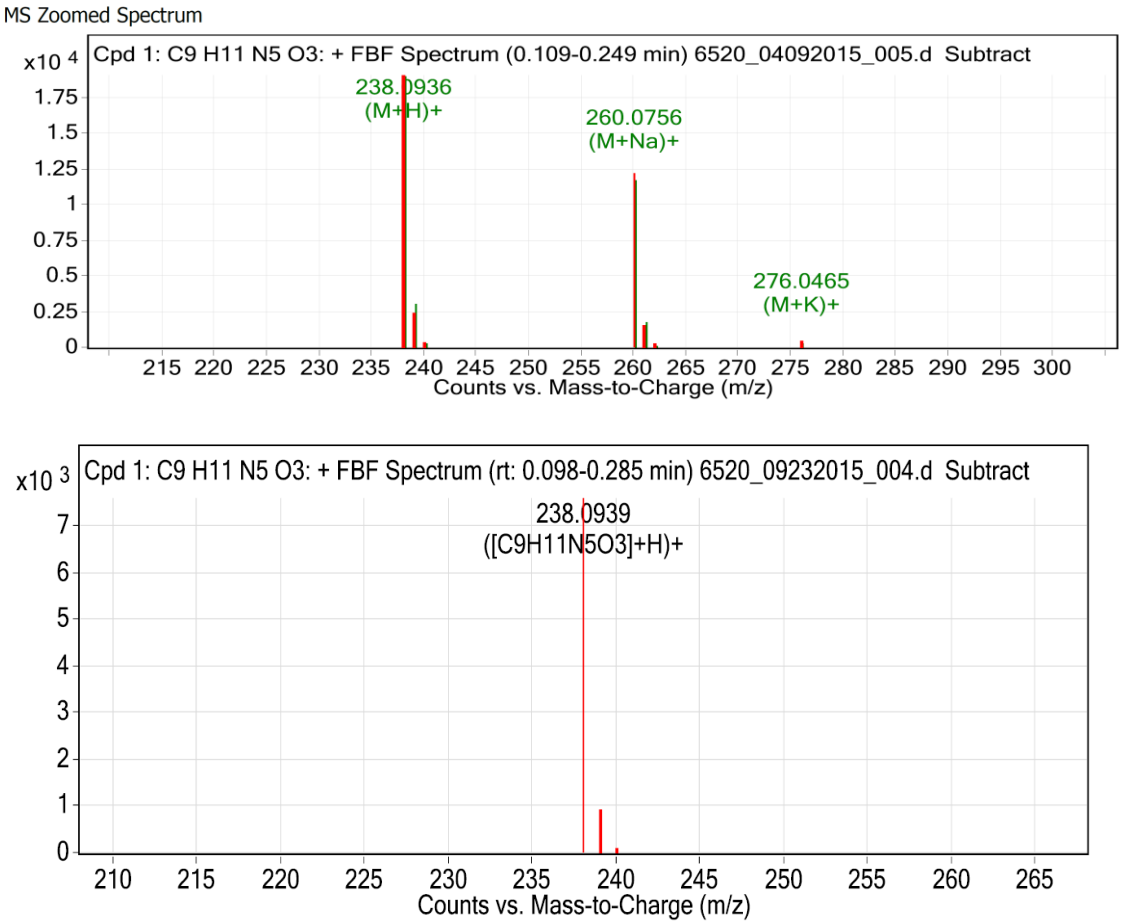


Fig. S1T HMBC spectrum of compound 4 in D_2O.

5. Figure S2. Comparison of ^{1}H NMR spectra of compound 1 in different conditions.

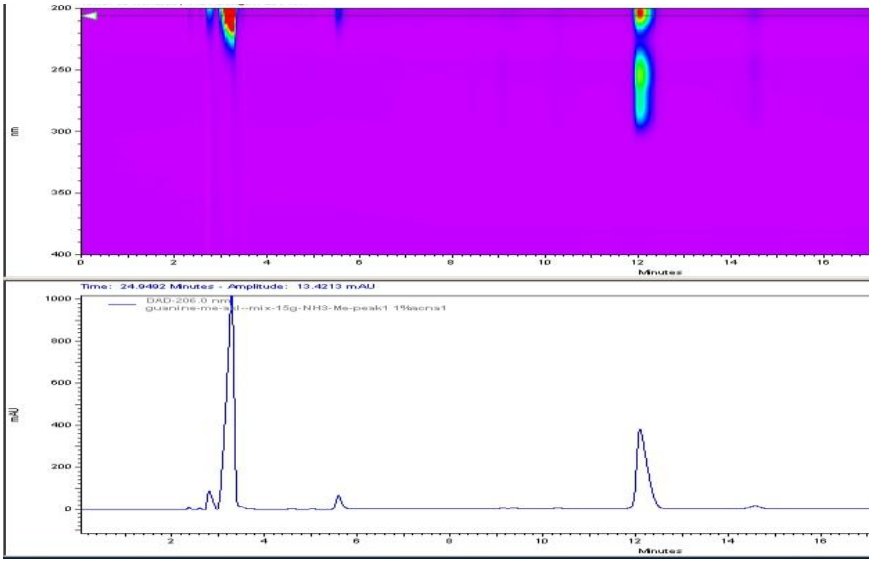


6. Figure S3. HRESIMS spectrum of compounds 1 and 2



7. Figure S4. The unusual venom duct of *C. genuanus*. Normally, the entire venom duct is just lightly pigmented as off-yellow. Here, only the distal part, closer to the venom bulb, is off-yellow. The proximal venom duct, closer to the proboscis of the snail, is unusual and is darkly pigmented

8. Figure S5. HPLC co-injection of natural and synthetic genuanine



0.4 mg of each natural and synthetic genuanine were mixed and dissolved in 1 mL H_2O, 1 uL of the solution was injected to HPLC (C18, 1% ACN in H_2O with 0,01% TFA) to obtain a single peak at 12.23 min.

9. Figure S6. Characteristics of crude venom extracts from *C. genuanus*

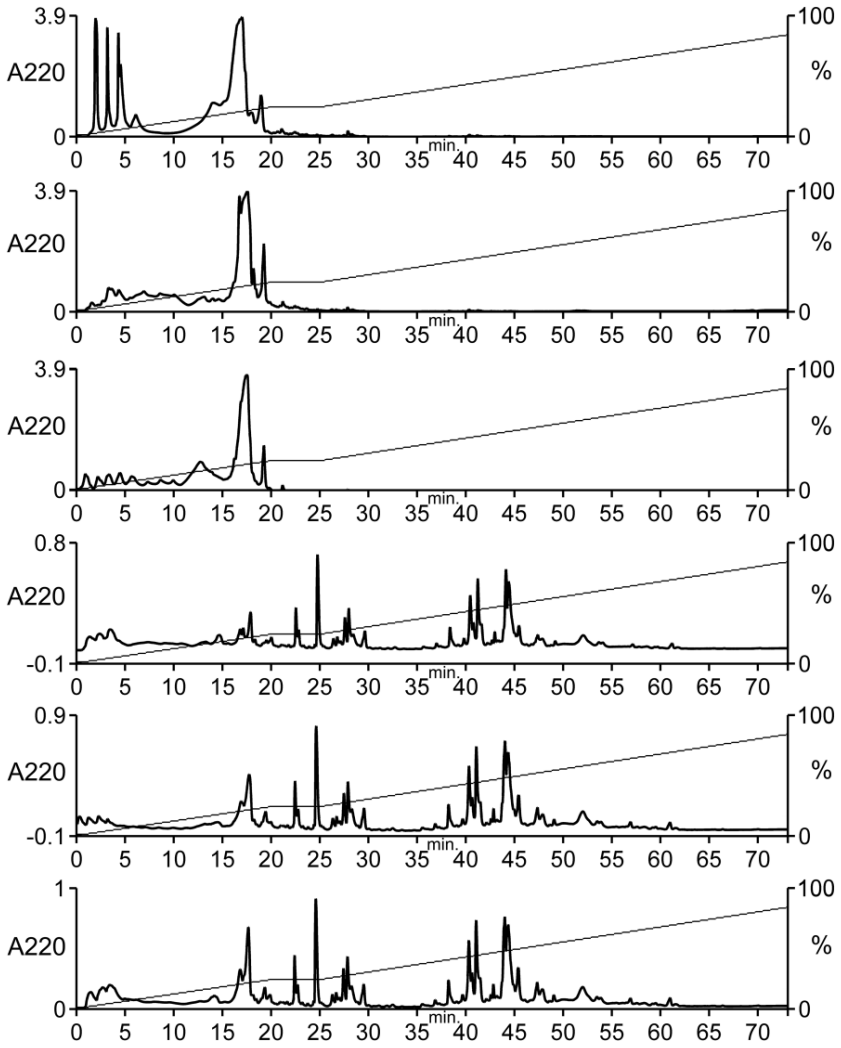


Fig. S6A. HPLC-UV of crude extracts from venom duct segments of 3 samples of *C. genuanus*. Top 3 traces are distal venom duct (red) segments, while bottom 3 are from proximal (yellow) venom ducts.

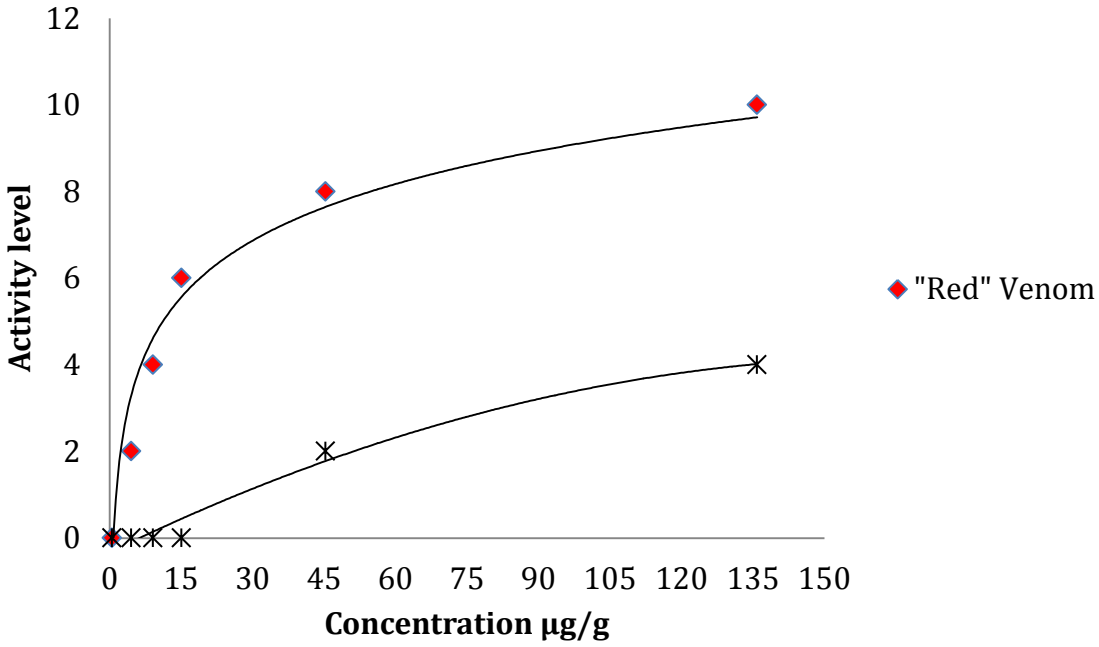


Fig. S6B. Activity of crude extracts in the mouse intracranial injection assay. These initial assays were performed for the purpose of identifying active components. Activity level 0, no effect; 2, mice don't move; 4, partial paralysis; 6-8, all limbs immobile, some stronger tremors; 10, strong tremors followed by death within seconds. N=1 per condition for these initial experiments (12 total mice).

10. References

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Chapter 5

Discussions and Conclusions

5. Discussion and Conclusions

Using different methodologies, such as, LC-MS, MALDI-TOF, and *De novo sequencing* new cone snail toxins from Cape Verde islands were identified. In terms of molecular mass, the distribution ranged from ~700 Da to ~5000 Da in most of the *Conus* species analyzed in this work – *C. crotchii*, *C. venulatus*, *C. speudonifier* and *C. trochulus* (Boa Vista Island), *C. ateralbus* (Sal Island) and *C. ermineus* (S. Antão Island). However, in terms of mass the *C. genuanus* (S. Vicente Island) have two different parts of venom. They were named the "red" and "yellow" portion of the venom duct. On the "yellow" part the mass identified are common for peptides in the *Conus* venom. Surprisingly, the "red" portion (one third of the venom duct) is rich on very small molecules with the mean of mass of 250 Da.

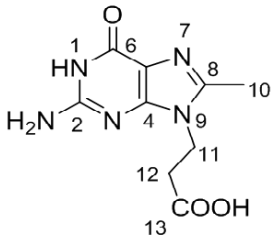
All the toxins isolated from Cape Verde cone snail are listed on Table 1 of this section. Twelve conopeptides were identified on *C. crocthii* (Chaper 2). Considered for validation the error between the experimental molecular mass (monoisotopic) and the calculated mass is less than 0.5 Da. In this article, the conopeptides detected by mass-matching (MSMS spectrum) belong to the A-, O1-, O2-, O3-, T- and D-superfamilies, which can block Ca^{2+} channels, inhibit K^{+} channels and act on nicotinic acetylcholine receptors (nAChRs). On *C. ateralbus* 5 news peptides, 4 of them unpublished yet, were identified. One of them is the peptide Atb-JI, which belongs to α-conotoxin pharmacology family. The α-conotoxins are considered competitive antagonist of acetylcholine (Ach) receptors. On the other hand, we identified a δ-conotoxin family, called δ-conotoxin AtVIA (Chaper 3). It should be stated that this is the first collection description and the first toxicological characterization of these species. The δ-conotoxin AtVIA have an excitatory activity that acted on a majority of mouse lumbar dorsal root ganglion neurons. AtVIA (potent in vertebrate systems; act on vertebrate Na channels) has conserved sequence elements when compared to δ-conotoxins from fish-hunting *Conus* species, and from a peptide purified from *Conus tessulatus*, a specie in the worm-hunting Indo-Pacific clade *Tessiliconus*. In contrast, there is no comparable sequence similarity with δ-conotoxins from the venoms of molluscivorous *Conus* species.

The unexpected results came from non-endemic *Conus*, *C. genuanus* (S. Vicente Island, Cape Verde), which have also been demonstrated on *C. imperialis* (Oahu, Hawaii) (Chaper 4). Surprisingly, instead of purifying a peptide (common in *Conus*), it was purified a novel small-molecule, a guanine derivative with unprecedented feature,

named genuanine (*genuanus* + guanine). Genuanine causes paralysis in mice, indicating that the small molecule, and not just peptides, may contribute to the biological activity of cone snail venom.

Table 1: Toxins isolated from Cape Verde cone snail venom

Conus Species	Sequence	Name	Methodology	Pharmacology Family
C. ateralbus	qCGADGQFCFLPGLGLNCCSGLCLLVCLPS	AtVIA[a]	*De novo* sequencing	δ-conotoxin
	EGCCSHPACSVNHPEICW	Atb-JI[a,b]		δ-conotoxin
	SFTMTAHPHGVHAYG[L/I]TS	at18a[a,b]	*De novo* sequencing/ Peaks Studio 5.3 software	unknown
	[L/I]FDG[L/I] [L/I]GQ[L/I]	at9b[a,b]		unknown
	[L/I]VST[L/I]FN[K/Q][L/I]G[K/Q][L/I]HH	at14c[a,b]		unknown
C. crotchii	ECCEDGWCCTAAPLTGR	TxVA	MSMS - MASCOT search/ UniProtKB	ϵ-conotoxin
	KRIPYCGQTGAECYSWCKEQHLIRCCDFVKYVGMNPPADKCR	im23.3		unknown
	QCTPKDAPCDDNNQCCSGLECKCFNMPDCQSGSTCRV	Ec15a		unknown
	PECCSDPRCNSTHPELCGGRR	Ai1.2		α-conotoxin
	ASEGCRKKGDRCGTHLCCPGLRCGSGRAGGACRPPYN	Bu2		unknown
	TCCKFQFLNFCCNEK	Ca5.1		unknown
	GCCSRPPCALSNPDYCG	PnMGMR-02		α-conotoxin

Conus Species	Sequence	Name	Methodology	Pharmacology Family
	RREVVTEE**C**EEYCKEQNKT**CC**GLTNGRPR**C**VGV**C**FG	VnMSGL-0123		unknown
	TRSGGA**C**NSHDQ**CC**INF**C**RKATST**C**M	Eb6.18		unknown
	S**C**GRRGKP**C**P**CC**RGFR**C**TGSF**C**RKWQ	Leo-O2		unknown
	CRIPNQK**C**FQHLDD**CC**SRK**C**NRFNK**C**V	PVIIA		κ-conotoxin
	DDESE**C**IINTRDSPWGR**CC**RTRM**C**GSM**CC**PRNG**C**T**C**VYHWRRGHGRS**C**PG	VxXXB		α-conotoxin
C. genuanus		genuanine[a]	NMR	unknown

a = new toxin; b = not published.

The discovery of a small active molecule on *Conus* venom (*C. genuanus* and *C. imperialis*) open new doors on pharmacological interest for toxins from venomous cone snails. Despite genuanine has unknown target (continues under investigation), natural small molecule derivatives of nucleic acids, such as cytosine arabinoside, often have novel biological activity, sometimes with important biomedical applications. Genuanine has mass less than 500 Da and is neuroactive on mice (see Chaper 4). These proprieties are very important for biomedical investigation. Should be highlight that *C. genuanus* belongs to a small number of cones with a pigmented venom duct, prompting us to search for very small molecule. This discovers extends our understanding of the chemical diversity of venom components.

In addition to the purification of new toxins in *C. ateralbus* and *C. genuanus*, the research was extended to more two Islands (S. Antão and Boa Vista), including in total, two non endemic species (*C. genuanus* and *C. ermineus*) and five endemic species (*C. ateralbus, C. crotchii, C. speudonifier, C. venulatus* and *C. trochulus*). It should be noted that to know the bioactivity profile we used Calcium-imaging studies on dissociated dorsal root ganglion (DRG) neurons and intracranial injection in mice (Table 2). The results show a diversity of bioactivity. In mice the strongest effect belongs to *C. ateralbus* (endemic – worm hunting), *C. venulatus* (endemic – worm hunting), *C. genuanus* (non-endemic – worm hunting) and *C. ermineus* (non-endemic – fish hunting). More specifically, DRG neurons results elicited amplification, block effect and excitatory phenotype, probably independent of the habitat and type of prey. Notably, Cape Verde cone snail venom may prove to have distinct molecular targets within a general class of receptors or ion channels.

Table 2: Toxicological venom effects from different Cape Verde cone snail using DRG neurons and mice assay

Island	*Conus* Species (toxin)	DGR Neurons (Cells %)	Mice Assay
Sal	*C. ateralbus* (AtVIA)	Indirect amplification (94.1)	Lethargic
	C. ateralbus (Atb-JI)	Indirect amplification (10.0)	Strong itch followed by death
	C. ateralbus (peptide candidate)	Block effect (45.9)	Not tested
	C. ateralbus (at18a)	Negative	Difficulty to walk, shivering and back legs drags
Boa Vista	*C. ateralbus* (at9b)	Negative	Difficulty to walk and back legs drags
	C. ateralbus (at14c)	Negative	Hyperactivity
	C. crotchii (crude venom)	Amplification (90.0)	Difficulty to walk
	C. speudonifier (crude venom)	Excitatory effects (30.0)	Hyperactivity and seizures
	C. venulatus (peptide candidate)	Not tested	Strong shivering, back legs drags, itch paralysis and death
S. Vicente	*C. trochulus* (crude venom)	Excitatory effects (90.0)	No effects
	C. genuanus (genuanine)	Negative	Difficult to walk and shivering
	C. genuanus (“red” crude venom)	Amplification (90.0)	Strong shivering followed by death
	C. genuanus (“yellow” crude venom)	Block effect (4.4), amplification (20.4)	Shivering, barrel rolling, paralysis, death
S. Antão	*C. ermineus* (crude venom)	Strong excitatory effects (90.0)	Strong shivering followed by death

In this work, we demonstrated that venom components (toxins) in Cape Verde cone snails (*Conus* spp.) are news peptides, some of them with high similarity with conopeptides already discovery. However, it should be note that, the study of a non-endemic species, *C. genuanus*, demonstrated for the first time the isolation of a novel small-molecule guanine derivative with unprecedented features, genuanine. Genuanine causes paralysis in mice, indicating that small molecules and not just polypeptides may contribute to the activity of cone snail venom. It was characterized the peptide profile from *C. crotchii* venom (endemic specie, collected on Boa Vista island). A large number of components were detected and some of them by matching with known peptide sequences from UniProtLB protein sequence database may have potential pharmacological activity. In the study of the *C. ateralbus* venom, an endemic cone snail that has been found only on the west side of the island of Sal, in the Cape Verde Archipelago off West Africa***,*** it we demonstrated that the new venom peptide with 30AA, δ-conotoxin AtVIA, has an excitatory activity that acted on a majority of mouse lumbar dorsal root ganglion neurons (DRG). Also, we provide the mass and toxicological profile of other Cape Verde cone species, *C. ermineus* (non-endemic), *C. pseudonivier* (endemic), *C. trochulus* (endemic) *and C. venulatus* (endemic). The global results shows that the venoms of Cape Verde cone species are rich source of powerful bioactive molecules in vertebrate systems and clearly with stronger pharmacology interests.